STUDIES ON TRANSAMINATION IN RAT SMALL INTESTINE

by

William Paranchych

Department of Biochemistry

September 1958

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STUDIES ON TRANSAMINATION IN RAT SMALL INTESTINE

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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DEPARTMENT OF BIOCHEMISTRY

by

William Paranchych

EDMONTON, ALBERTA

September, 1958.

ABSTRACT

Glutamic-oxalacetic transaminase (GOT) levels in the small intestine of the rat were found to be apportioned approximately evenly along the entire length of the intestine. Levels of glutamic-pyruvic transaminase (GPT), however, were shown to be greatest from the third to the sixth sections (measured in 10 cm. lengths from the pylorus), and a considerable drop of activity was shown to occur in the terminal 30 cm.

Kinetic studies of intestinal GOT and GPT by a suitable colorimetric method showed that both enzymes possess maximum catalytic activity at pH 8.5 in the presence of 0.10 M phosphate buffer. Repetition of the experiment by means of a precise spectrophotometric assay procedure confirmed this finding for GOT. When barbital buffer was used, however, GOT was shown to have a pH optimum of 9.08. The reaction was shown to be of zero order when a substrate concentration of 60 micromoles of amino acid and 20 micromoles of α-ketoglutaric acid per ml. reaction mixture was employed. An enzyme concentration of 0.2 ml. homogenate per 1.5 ml. reaction mixture and a reaction time of 30 minutes at 37°C. were also required for a zero order reaction. Over the range 5-40°C. the energies of activation were 8,800 calories per mole for GOT and 9,170 calories per mole for GPT.

The mean values of GOT and GPT in the third 10 cm. section of intestine of non-fasting rats were, respectively, 718 $\pm$ 23 and 894 $\pm$ 27 units per gm. wet intestine. Fasting caused the transaminase levels to drop rapidly for the first two days and then more gradually thereafter. Simultaneous estimation of the concentrations of aspartic acid, glutamic acid, glycine, and alanine in the second 10 cm. section showed that the levels of these amino acids decreased gradually during four days of fasting.

Force-feeding of 0.75 M glycine solutions to two-day fasted rats resulted in a decrease of intestinal GOT and GPT activity. A simultaneous loss of glutamic acid led to the hypothesis that intestinal absorption of glycine is followed by synthesis of glutathione, and that γ-glutamylcysteine, which is an intermediate product in glutathione synthesis, is responsible for inhibition of GOT and GPT. An increase of aspartic acid concentration was found when 0.75 M L-alanine was fed to fasted rats. This was explained on the basis of transamination and involvement of the TCA cycle. Loss of GOT and GPT activity again was attributed to inhibition by γ-glutamylcysteine. When L-glutamic and L-aspartic acids were fed, evidence was obtained in support of previous work that these amino acids undergo transamination in rat intestine. The decrease of glycine concentration which occurred was presumed to be the result of pyruvate requirement for the transaminase reactions.

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CHAPTER I

GENERAL INTRODUCTION

Transamination is a reversible enzyme-catalyzed transfer of the α-amino nitrogen of an amino acid to an α-keto acid which results in the synthesis of a second amino acid and a new α-keto acid. This type of chemical conversion was first described in 1937 by Braunstein and Kritzmann (16), who used pigeon breast muscle as the source of transaminase and postulated that the reaction could occur with any amino acid (except glycine) and α-ketoglutarate or oxalacetate. This conclusion was later modified (13) following evidence provided by Cohen (30) for the existence of only two transaminating systems in swine heart muscle:

(1) Oxalacetate + L-glutamate $\rightleftharpoons$ L-aspartate + α-ketoglutarate

(2) Pyruvate + L-glutamate $\rightleftharpoons$ L-alanine + α-ketoglutarate

Subsequently, evidence for additional transaminases in heart muscle, liver, and kidney was obtained by Cohen and Cammarata (21). Extracts of these tissues, with added pyridoxal phosphate, were shown to be capable of transferring α-amino groups of 25 different α-amino acids to α-ketoglutaric acid to form glutamic acid. A great deal of evidence has since accumulated, and it is now recognized that virtually all the natural amino acids may participate in transamination.

The following nomenclature has been generally accepted for the two transaminase reactions which were described by Cohen (30):

(a) L-glutamate + oxalacetate $\rightleftharpoons$ α-ketoglutarate + L-aspartate
Glutamic-oxalacetic transaminase (GOT) or glutamic-aspartic transaminase

(b) L-glutamate + pyruvate $\rightleftharpoons$ α-ketoglutarate + L-alanine
Glutamic-pyruvic transaminase (GPT) or glutamic-alanine transaminase

Transaminases have been found in a great variety of plant and animal tissues and microorganisms (93). In 1952, Awapara and Seale (7) showed that GOT and GPT are present in eight organs of the rat. Greatest GOT activity was shown in heart muscle homogenates, followed in decreasing order by skeletal muscle, lung, brain, liver, spleen, prostate, and testes. Karmen, Wroblewski, and LaDue (66) found that red-cell hemolysates of human blood contained approximately ten times as much GOT and five times as much GPT activity as did the sera. The GOT and GPT activity of tumours has been shown by numerous investigators to be the same as or lower than, that of normal tissues (13, 32, 33, 71).

The use of the classical methods of fractional precipitation with salts and organic solvents has led to the development of several methods for the purification of

GPT (53) and GOT (53, 111, 130, 23) of pig heart. Recently, use has been made of column chromatography for preparation of transaminases. Jenkins and Sizer (64) reported the preparation of pig heart GOT of 70% purity. Pyridoxal phosphate was found to be firmly bound to the enzyme and spectral studies at various pH values suggested that the aldehyde group was not free but presumably joined in imine linkage to an amino group of the enzyme. Lis (82) also purified pig heart GOT by column chromatography. She achieved a fifty-fold purification and reported that the enzyme obtained was the holoenzyme.

It is now clearly established that vitamins B_6 are required for transamination. Schlenk and Snell (131) and others (80, 18, 39, 96) have provided abundant evidence that deficiency of vitamins B_6 is associated with reduced tissue transaminase activity. In addition, all purified preparations of pig heart GOT, whether obtained by salt fraction or column chromatography, were shown to contain pyridoxal phosphate. In most cases, pyridoxal phosphate has been shown to be the active coenzyme. However, Meister and his associates (95, 97) have provided evidence that pyridoxamine phosphate can replace pyridoxal phosphate as a cofactor for the GOT of pig heart. Recently, Meister and Downey (94) found that administration to rats of isonicotinic acid hydrazide, led to a marked reduction in the activities

of both the liver glutamic-pyruvic and glutamine-α-keto acid transaminase systems. Addition to tissue extracts *in vitro* of either pyridoxal phosphate or pyridoxamine phosphate restored the activities of both enzymes to approximately normal values. This suggests that isonicotinic acid hydrazide either replaces the coenzyme or interferes with its synthesis.

The recognition of the function of vitamins B_6 in the transaminase reaction was an important step forward in understanding the mechanism of enzymic transamination. In 1945, Snell (136) made the interesting observation that heating pyridoxal with glutamic acid leads to the formation of pyridoxamine and α-ketoglutaric acid. The reverse reaction was also found to occur. This led to the suggestion by Schlenk and Fisher (130) that pyridoxal phosphate and pyridoxamine phosphate act as intermediates in biological transamination by means of a Schiff base mechanism. Excellent support for this mechanism has been provided by several investigators (100, 83, 55) who demonstrated that metal ions, which function in the formation of chelate rings, are necessary for the reactions. By means of chromatographic and electrophoretic methods, Fasella and his associates (43) have identified and isolated two compounds intermediate in nonenzymic transamination. The spectra and chemical properties of these compounds indicate that they are

Schiff-base metal chelates between pyridoxal or pyridoxal phosphate and amino acids, and pyridoxamine or pyridoxamine phosphate and α-keto acids.

Hilton, Barnes, Henry, and Enns (58) have shown that deuterium is rapidly incorporated into aspartate and glutamate when the GOT transaminase reaction is carried out in the presence of D_2O. They suggested that the rapid hydrogen exchange of aspartate and glutamate is closely associated with the action of the enzyme.

Possibly one of the most important metabolic roles of transamination that have been established is the reversible deamination mechanism proposed by Braunstein and coworkers (13, 14, 15). The mechanism, which involves a coupled reaction between an α-ketoglutarate-amino acid transaminase and the glutamic dehydrogenase system, represents a significant link between the metabolism of amino acids and carbohydrates, and also provides a pathway for the conversion of α-amino acids to ammonia and other nitrogen-containing products. In addition to the significant role of transamination in the interrelationships between various amino acids and the tricarboxylic acid cycle, Ratner (118) has suggested that transamination leading to aspartate formation may be a key reaction in the control of urea formation. Considerable attention has been directed toward the possible role of transamination in protein synthesis and growth,

(2, 13, 32, 81, 135) but the studies thus far do not seem to have established any consistent relationship between transaminase activity and protein synthesis.

Gavosto, Pileri, and Brusca (50) have reported that when near-toxic doses of cortisone are administered to rats for three days, significant increases of GOT and GPT activities are found in the rat livers. They suggested that cortisone increases gluconeogenesis and imposes a negative nitrogen balance by enhancing transamination processes. Independently, Rosen and coworkers (123) showed that when rats were treated with hydrocortisone, GPT activity in livers increased as high as 500 per cent. They postulated that the control of hepatic levels of GPT by glucocorticosteroids is related to the mechanism by which these compounds exert their gluconeogenic activity.

Considerable investigation has been carried out concerning the role of transamination in the metabolism of certain individual amino acids. Enzymes catalyzing the transamination of kynurenine (87, 88, 63), histidine (3, 4), serine (127), and cysteine (69) are attracting increasing attention.

The finding that GOT and GPT exist in high concentration in animal tissues (31, 7) has led numerous investigators to determination of transaminase activity in human serum as a diagnostic aid. LaDue, Wroblewski, and Karmen (77),

and later others (27, 84, 148) have reported on striking transient elevations of serum glutamic-oxalacetic transaminase activity in acute myocardial infarction. Elevations of serum GOT and GPT activity also have been found to occur in hepatic disorders (102, 27, 152), muscular dystrophy (115, 120), and cerebral infarctions (54). The elevated serum transaminase levels apparently are due to leakage of the enzymes from damaged tissues.

Despite the widespread interest in the role of transamination in metabolism, relatively few studies have been carried out concerning transamination in mammalian intestine. Workers in this laboratory (141) have recently completed an investigation on the effect of amino acid ingestion on levels of alkaline phosphatase in rat intestine. Several interesting clues to the mechanism of intestinal absorption of amino acids arose out of the investigation. The following study, which deals with transamination in the small intestine of the rat, was undertaken with the hope of providing further information on the mechanism of intestinal absorption of amino acids.

A. The Assay of Intestinal Glutamic-Oxalacetic and Glutamic-Pyruvic Transaminases

1. Review of Available Methods

Since the discovery of the transaminase reaction by Braunstein and Kritzmann (16), a variety of analytical methods for the estimation of the activity of various transaminases has appeared in the literature. As stated previously, the glutamic-oxalacetic and glutamic-pyruvic transaminase systems involve the following reactions:

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{GOT}} \text{oxalacetate} + \text{glutamate}$$

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{GPT}} \text{pyruvate} + \text{glutamate}$$

The activity of either of these transaminases, therefore, may be estimated by incubating the enzyme preparation with the appropriate pair of substrates under suitable conditions and then measuring either the disappearance of one or both of the substrates, or the appearance of one or both of the products.

The earlier methods involved manometric techniques which usually were difficult and cumbersome. One of the first suitable assays was that of Cohen (29), in which the transaminase reaction was followed by the measurement of changes in glutamic acid concentration. The glutamic acid was first oxidized by chloramine-T to β-cyanopropionic acid

and the latter was hydrolyzed to succinic acid. The succinic acid was then determined manometrically with the aid of the succinoxidase preparation which had been described by Krebs (73). Chloramine T again was employed by Cohen (30) in 1940 when he reported a procedure for transaminase estimation by measurement of aspartic acid. This procedure was based on the finding of Dakin (36) that in the presence of an excess of chloramine T, aspartic acid yields two molecules of CO_2 while most of the other amino acids yield only one. Green, Leloir, and Nocito (53) estimated transaminase activity by manometric measurement of α-ketoglutaric acid. The α-ketoglutarate was first converted to succinate with hydrogen peroxide and the oxygen uptake was then measured after the addition of succinic dehydrogenase. Several workers have studied the transaminase reaction by means of a manometric estimation of oxalacetic acid (5, 30, 53, 79). The method depends upon the decarboxylation of oxalacetic acid with a solution of aniline citrate. Perhaps the most accurate manometric techniques for determining transaminase activity have been those employing specific bacterial amino acid decarboxylases (145, 79, 21, 44, 103, 99, 74, 121, 122). Because of their specificity, these methods allow accurate estimations to be made of glutamic acid and of aspartic acid.

Cook (34) assayed glutamic-oxalacetic transaminase activity by titrimetric measurements of the carbon dioxide

yield in the ninhydrin reaction with α-amino acids. He found that under controlled conditions ninhydrin removed both carboxyl groups from aspartic acid but only the α-carboxyl groups from glutamic acid. The difference in CO_2 production was thus used as a measure of transamination.

The use of quantitative paper chromatography for assaying transaminase activity has the advantage that all four components of the reaction may be determined simultaneously. Such a technique is especially desirable when crude enzyme preparations are being investigated. Although paper chromatographic methods lack a high degree of accuracy, and usually are time-consuming, numerous investigators have recently utilized this technique for the investigation of transaminase reactions (38, 44, 45, 46, 52, 59, 117, 121, 124, 66, 7, 144).

Spectrophotometric measurement of the substrates or products as a means of determining glutamic-oxalacetic transaminase activity was first suggested by Green et al. (53) and also has been utilized by several others (107, 108, 109, 7, 23, 24, 12, 35). At a wavelength of 280 millimicrons oxalacetic acid possesses a high extinction coefficient in comparison to the other three components of the reaction system. It is thus possible to follow the glutamic-oxalacetic transaminase reaction by measuring the increase in opticaldensity which occurs as oxalacetate is

formed. Karmen, Wroblewski, and LaDue (66) described a spectrophotometric method for the assay of glutamic-oxalacetic transaminase activity which involves the use of reduced diphosphopyridine nucleotide and malic dehydrogenase. A suitable enzyme preparation is added to aspartic and α-ketoglutaric acid and then, in the presence of an excess of malic dehydrogenase, the oxalacetate which forms in the course of the reaction is converted to malic acid. This reaction is coupled to an oxidation of reduced diphosphopyridine nucleotide and the oxidation reaction is followed by measuring the decrease in optical density at a wavelength of 340 millimicrons. By substituting alanine for aspartic acid, and lactic dehydrogenase for malic dehydrogenase, the Karmen method was modified by Wroblewski and LaDue (152) and it was utilized for assaying glutamic-pyruvic transaminase. Lowry, Roberts, and Chang (85) also employed the diphosphopyridine nucleotide principle for determining glutamic-oxalacetic transaminase activity. Instead of following the disappearance of reduced diphosphopyridine nucleotide at 340 millimicrons, however, they measured the appearance of the oxidized form of the nucleotide fluorometrically. They claimed that the fluorometric method was several thousand-fold more sensitive than the measurement of the reduced form of the coenzyme at 340 millimicrons. This also has been found to be true by others (62).

Along with the growing interest in the transaminase reaction there also developed a need for simple colorimetric methods by which large numbers of transaminase determinations could be performed in a relatively short period of time. Several reliable methods recently have been reported. Green et al. (53) described a procedure for the measurement of glutamic-pyruvic transaminase in which pyruvic acid was allowed to react with salicylaldehyde to form an orange-colored product. The absorption was then measured at a wavelength of 440 millimicrons. A colorimetric method for the estimation of glutamic-oxalacetic transaminase was first reported by Tonhazy, White, and Umbreit (140). Decarboxylation of oxalacetate to pyruvate with aniline citrate was employed and then, after the α-keto acids in the mixture had been converted to the 2,4-dinitrophenylhydrazones, the pyruvate hydrazone was extracted in toluene and colorimetrically measured in an alkaline solution. Although Tonhazy's 2,4-dinitrophenylhydrazine method has found more popularity than that of Green's salicylaldehyde method (20, 42, 19, 119, 126), it has the disadvantage of being less versatile. The 2,4-dinitrophenylhydrazine forms a hydrazone with α-ketoglutaric acid as well as with pyruvic acid and this results in high blank values. Serious difficulties are especially encountered when kinetic studies such as the investigation of the effect of substrate concentration are attempted. Salicylaldehyde, on the other hand, reacts much more specifically with pyruvate.

2. Methods Adopted

As this study was to involve large numbers of glutamic-oxalacetic and glutamic-pyruvic transaminase assays, it was decided that the selection of a simple colorimetric technique would be most feasible. At first, the 2,4-dinitrophenylhydrazine method of Tonhazy et al. (140) was tested, but, because of the lack of specificity of 2,4-dinitrophenylhydrazine for pyruvate and because this lead to difficulty in establishing optimum conditions, this procedure was rejected.

Consequently, a trial was made of the salicylaldehyde method of Green et al. (53). This procedure was found to be suitable, but, as Green et al. had utilized the method only for the assay of glutamic-pyruvic transaminase, it was necessary to adapt the technique so that it could be used also for the determination of glutamic-oxalacetic transaminase activity. Thus, following certain modifications, the salicylaldehyde method was finally chosen as the procedure which would be used for the assay of both glutamic-oxalacetic and glutamic-pyruvic transaminases in the small intestine of the rat.

It was realized, however, that this procedure was inadequate for the investigation of certain aspects of the kinetics of these two transaminases since it did not seem probable that, under non-optimal conditions, true initial velocities could be obtained with the colorimetric method.

Thus, the spectrophotometric procedure of Nisonoff and coworkers (107, 108), as utilized by Cook (35), was employed whenever it was thought necessary to verify the kinetic data obtained by means of the colorimetric method for glutamic-oxalacetic transaminase with data obtained by the more exact, spectrophotometric procedure. However, an analogous technique was not available for verifying glutamic-pyruvic transaminase kinetic data, because pyruvic acid and α-keto-glutaric acid possess almost identical absorption spectra.

3. Colorimetric Determination of Intestinal Glutamic-Oxalacetic and Glutamic-Pyruvic Transaminases

Reagents

1. Substrate for glutamic-oxalacetic transaminase assay

3.484 grams of K_2HPO_4, 1.844 grams L-aspartic acid and 0.675 grams α-ketoglutaric acid were dissolved in approximately 150 ml. of distilled water. The pH was adjusted to 8.5 with normal KOH and the volume was made up to 200 ml.

1.3 ml. of this reagent contains 90 micromoles L-aspartate and 30 micromoles α-ketoglutarate. The phosphate concentration is 0.10 molar.

K_2HPO_4: Anhydrous, Fisher Certified Reagent.

L-Aspartic Acid: Nutritional Biochemicals Corporation.

α-Ketoglutaric Acid: Sigma Chemical Corporation.

Potassium Hydroxide: Analar, The British Drug Houses Ltd.

2. <u>Substrate for glutamic-pyruvic transaminase assay</u>

3.484 grams K_2HPO_4, 0.675 grams α-ketoglutaric acid and 1.233 grams L-alanine were dissolved in approximately 150 ml. of distilled water. The pH was adjusted to 8.5 with normal KOH and the volume was made up to 200 ml.

1.3 ml. of this reagent contains 90 micromoles L-alanine and 30 micromoles α-ketoglutaric acid. The phosphate concentration is 0.10 molar.

L-Alanine: Nutritional Biochemicals Corporation.

3. <u>0.10 M Phosphate buffer, pH 8.5</u>

13.609 grams KH_2PO_4 was dissolved in approximately 900 ml. distilled water. The pH was adjusted to 8.5 with normal KOH and the volume was made up to 1000 ml.

KH_2PO_4: A.C.S., Allied Chemical and Dye Corporation.

4. <u>Trichloroacetic acid, 10% w/v</u>

Trichloroacetic Acid: Reagent, Merck and Co. Ltd.

5. <u>Aniline-citrate solution (140)</u>

5.0 grams citric acid was dissolved in 5.0 ml. distilled water. 5.0 ml. aniline was added.

Citric Acid: Analar, The British Drug Houses Ltd.

Aniline: Fisher Certified Reagent.

6. <u>Concentrated KOH reagent (53)</u>

500 grams KOH was dissolved in 300 ml. distilled water.

7. <u>Salicylaldehyde reagent, 2% v/v in 95% ethyl alcohol, (53)</u>

Salicylaldehyde: Redistilled, Eastman Kodak.

95% Ethyl Alcohol: Unmatured Hospital Spirits, Reliance Chemicals Ltd.

8. <u>Pyruvic acid standard solution (3.0 micromoles per ml.)</u>

33.0 milligrams of sodium pyruvate was dissolved in 0.10 M phosphate buffer, pH 8.5, and the volume was adjusted to 100 ml.

Sodium Pyruvate: Reagent, Nutritional Biochemicals Corp.

<u>The salicylaldehyde color reaction</u>

In the method of Green <u>et al</u>., one ml. of pyruvic acid solution was mixed with 1.0 ml. of concentrated KOH reagent and 0.5 ml. of 2% salicylaldehyde solution. After 10 minutes incubation at 38°C., the solution was made up to 25 ml. with water and the extinction at 440 millimicrons was determined against a blank which contained all additions except pyruvic acid. The calibration curve showed that Beer's Law was followed if the pyruvate concentration was kept within the range of 0 to 5 micromoles.

In this study, preliminary experiments were carried out in order to determine approximately how much transaminase activity exists in crude homogenates of rat intestine. It was found that when a homogenate which was approximately a 1:200 dilution of the intestine was used, one ml. of the reaction mixture which contained 0.133 ml. of this homogenate produced 0.3 to 0.7 micromoles of α-keto acid when it was incubated for 30 minutes at 37°C. The system contained a substrate concentration of 67 micromoles of aspartate or alanine and 15 micromoles of α-ketoglutarate per ml. and it

was buffered to pH 7.4 with 0.1 M phosphate buffer. Because the amount of pyruvate which was formed by this system was about one-fifth of the amount which was formed by the system of Green et al. (53), it was necessary to modify the colorimetric method for pyruvic acid in such a manner that greater sensitivity would be achieved.

The colorimetric method which was finally adopted for the estimation of pyruvic acid was the following.

After the transaminase reaction mixture had been incubated at 37°C. for 30 minutes, the reaction was stopped by the addition of 0.5 ml. of 10% trichloroacetic acid. The proteins were removed by centrifugation for 10 minutes at 3000 r.p.m. in an MSE clinical centrifuge and 1.0 ml. of clear supernatant solution was transferred to a photometer tube. To this, with mixing after each addition, was added 1.0 ml. of saturated KOH and 0.3 ml. of 2% salicylaldehyde reagent. The solution was incubated at 37°C. for 10 minutes and then it was mixed with 3.0 ml. of distilled water. The absorption was determined immediately in a Klett-Summerson photometer using a KS-44 filter against a blank which was identical to the test solution except that 0.1 M phosphate buffer had been substituted in place of the homogenate.

In order to determine the stability of the final orange-colored solution, the color reaction was performed on blank and pyruvate-containing solutions for both the glutamic-

was buffered to pH 7.4 with 0.1 M phosphate buffer. Neither the amount of tyrosine which was formed in this system [illegible] was large enough to be determined by the method of Udenfriend et al. (33), it was necessary to modify the colorimetric method for tyrosine so that a greater sensitivity would be acquired.

The colorimetric method which was finally adopted for the estimation of tyrosine was the following.

After the incubation reaction mixture had been incubated at 37°C. for 30 minutes, the reaction was stopped by the addition of 0.5 ml. of 30% trichloroacetic acid. The proteins were removed by centrifugation for 10 minutes at 3000 r.p.m. in an IEC clinical centrifuge and 1.0 ml. of clear supernatant solution was transferred to a colorimeter tube. To this, with mixing after each addition, was added 1.0 ml. of [illegible] and 0.5 ml. of [illegible] reagent. The solution was incubated at 37°C. for 10 minutes and then it was mixed with 3.0 ml. of distilled water. The absorption was determined immediately in a Klett-Summerson photometer using a [illegible] filter against a blank which was identical to the test solution, except that 0.1 M phosphate had been substituted in place of the homogenate.

In order to determine the stability of the final reaction solution, the color reaction was performed on tyrosine-containing solutions for [illegible]-

oxalacetic and glutamic-pyruvic systems and then absorption readings were determined at five minute intervals for a period of 90 minutes. The measurements were made in a KS photometer with a KS-44 filter. The test solution contained 60 micromoles of aspartic acid or alanine, 20 micromoles of α-ketoglutaric acid and 0.4 micromoles of pyruvic acid per ml. of reaction mixture and it was buffered to pH 8.5 with 0.1 M phosphate buffer. The blank solution was the same as the test solution except that pyruvic acid was omitted. A pyruvate concentration of 0.4 micromoles per ml. was chosen because this was approximately of the same magnitude as the pyruvate concentration which was encountered in the majority of transaminase assays. To 1.5 ml. of the solution was added 0.5 ml. of 10% trichloroacetic acid and the procedure was carried out exactly as described previously. The results of the experiment are presented in Table I and the values shown are the average of four determinations in which replicates agreed within five KS units. The values shown in Table I are presented graphically in Figs. 1 and 2. It was found that the color of the blank solutions of both the GOT and GPT systems began increasing after twenty minutes. The test solution of the GOT system remained unchanged for forty minutes before showing marked increases of absorbancy and the test solution of the GPT system remained constant for twenty five minutes before showing increases of absor-

TABLE 1

The Relationship Between Absorbancy and Time for Colored Solutions Obtained by the Salicylaldehyde Method for Pyruvic Acid.

	Photometer Reading in KS Units of Solutions Containing GOT Substrate			Photometer Reading in KS Units of Solutions Containing GPT Substrate		
Time in Minutes	Blank Solution	Test Solution (0.4 μM Pyruvate per ml.)	Test Solution Read Against Blank Set at Zero.	Blank Solution	Test Solution (0.4 μM Pyruvate per ml.)	Test Solution Read Against Blank Set at Zero.
5	30	109	79	35	100	65
10	30	109	79	36	100	64
15	31	109	78	37	100	63
20	32	109	77	38	100	62
25	35	109	74	42	100	58
30	37	109	72	44	104	60
35	39	109	70	47	106	59
40	42	109	67	49	109	60
45	43	110	67	52	111	59
50	45	112	67	55	113	58
55	47	114	67	58	116	58
60	50	116	66	61	118	57
65	52	117	65	63	120	57
70	54	119	65	66	122	56
75	56	120	64	69	125	56
80	59	122	63	72	127	55
85	60	124	64	74	129	55
90	63	127	64	77	131	54

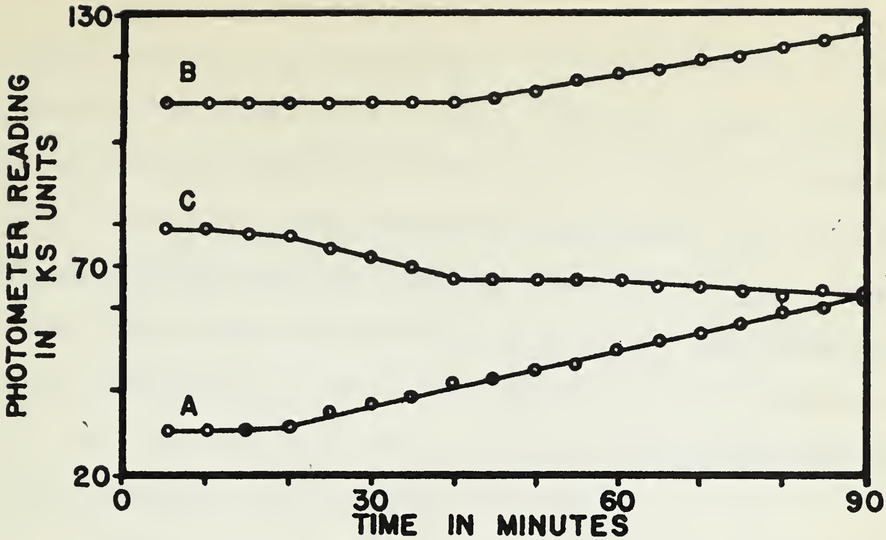

Fig. 1. The Relationship Between Absorbancy and Time For Colored Solutions Obtained by the Salicylaldehyde Method for Pyruvic. (GOT System).

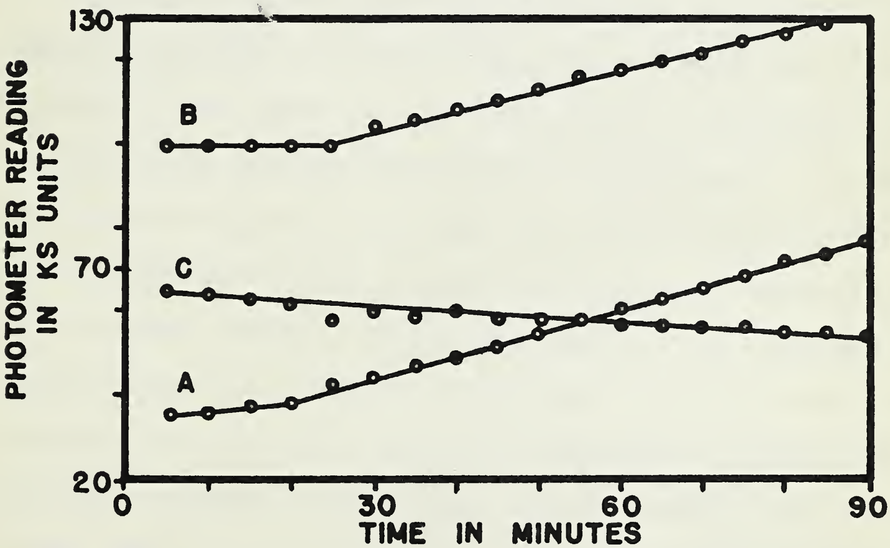

Fig. 2. The Relationship Between Absorbancy and Time for Colored Solutions Obtained by the Salicylaldehyde Method for Pyruvic Acid. (GPT System).

Legend for Figs. 1 and 2.
A: Blank Solution.
B: Test Solution Containing 0.4 µM Pyruvate per ml.
C: Test Solution Read Against Blank Set at Zero.

bancy. When the test solutions of each system were read against the blanks set at zero, however, their absorbancies were found to remain constant for the first ten minutes and then they were found to decrease gradually for the remainder of the ninety minutes. As a consequence of this experiment, blank solutions subsequently were set at zero immediately upon completion of the color reaction and the absorbancies of the test solutions were measured within ten minutes of the completion of the color reaction.

Green <u>et</u> <u>al</u>. (53) showed that maximum absorption of colored solutions obtained by the salicylaldehyde method for pyruvic acid occurred at a wavelength of 440 millimicrons. In order to confirm this information, the salicylaldehyde color reaction was carried out on substrate solutions of the GOT and GPT systems containing 0.4 and 0.8 micromoles of pyruvic acid per ml. of reaction mixture and then absorption curves of these solutions were determined in a Beckman Model DU spectrophotometer. The time which was required to run a complete absorption curve was about twelve minutes. In order to account for changes of absorbancy which may have occurred in the blank solution during the twelve minute period, two absorption curves were determined for each substrate system: one in which readings were taken in the direction of increasing wavelengths, and the other in which readings were taken in the reverse direction. As the

absorption curves of both the GOT and GPT systems were found to be identical, the four determinations, including two determinations for each system, were grouped together and the mean values which were obtained are presented in Table II. In the region of the absorption curve of highest absorbancy replicate values agreed within 3% transmittance units. It is evident from Fig. 3 that the area of highest sensitivity of colored solutions obtained by the salicylaldehyde color reaction for pyruvic acid is in the region of 440 to 460 millimicrons and that maximum absorption occurs at a wavelength of 443 millimicrons. Subsequent colorimetric transaminase estimations, therefore, were determined in a KS photometer with a KS-44 filter having transmittance limits of 410 to 480 millimicrons.

Calibration curves for pyruvic acid for each transaminase system were prepared by performing the salicylaldehyde color reaction on a series of solutions containing 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75, and 2.0 micromoles pyruvic acid per 1.5 ml. of incubation mixture. The mixture contained 90 micromoles of aspartate or alanine and 30 micromoles α-ketoglutarate per 1.5 ml. and it was buffered to pH 8.5 with 0.1 M phosphate buffer. Although the calibration curve for the GOT system occasionally was found to have a slightly greater slope than the calibration curve for the GPT system, in most determinations the calibration

curves for the two systems were found to be identical. Standard solutions containing 0.6 micromoles pyruvic acid per 1.5 ml. of reaction mixture were determined along with each set of transaminase assays and the calculations were based on the values which were obtained for these standard solutions. A new pyruvic acid standard solution was prepared once a week. The results which were obtained for the calibration curve are presented in Table III and the curve is shown graphically in Fig. 4.

Higher absorbancy values were obtained with pyruvic acid standard solutions than with standard solutions of pyruvic acid containing α-ketoglutarate and aspartate or alanine. This information suggested that one or more of the transaminase substrates was affecting the color reaction. To investigate this further, solutions of aspartic acid, glutamic acid, alanine, pyruvic acid, oxalacetic acid, and α-ketoglutaric acid each were prepared to contain 0.5, 1.0, and 1.5 micromoles per 1.5 ml. A blank of distilled water was included and then the solutions were submitted to the salicylaldehyde color reaction. The color reaction also was performed on pyruvic acid solutions which contained 90 micromoles of aspartate or alanine and 30 micromoles of α-ketoglutarate per 1.5 ml. A plot (Fig. 5) of the results, which are presented in Table IV, shows that all of the amino and α-keto acids which are involved in the transaminase

curves for the two systems were found to be identical. Standard solutions containing 0.5 micromoles pyruvic acid per 1.5 ml. of reaction mixture were determined along with each set of transaminase assays and the calculations were based on the values which were obtained for these standard solutions. A new pyruvic acid standard solution was prepared once a week. The results which were obtained for the calibration curve are presented in Table (3) and the curve is shown graphically in Fig. 1.

Higher absorbency values were obtained with pyruvic acid standard solutions than with standard solutions of pyruvic acid containing a-ketoglutarate and aspartate or alanine. This difference suggested that one or more of the transaminase substrates was affecting the color reaction. To investigate this problem, solutions of aspartic acid, glutamic acid, alanine, pyruvic acid, oxalacetic acid, and a-ketoglutaric acid each were prepared to contain 0.5, 1.0, and 1.5 micromoles per 1.5 ml. A blank of distilled water was included and then the solutions were subjected to the salicylaldehyde color reaction. The color reaction also was performed on pyruvic acid solutions which contained 50 micromoles of aspartate or alanine and 20 micromoles of a-ketoglutarate per 1.5 ml. A plot (Fig. 2) of the results, which are presented in Table (4), shows that all of the amino and a-keto acids which are involved in the transaminase

TABLE II

The Relationship Between Absorbancy and Wavelength of Colored Solutions Obtained by the Salicylaldehyde Method for Pyruvic Acid.

	Optical Density	
Wavelength in Millimicrons	Pyruvate Conc. = 0.40 µM per ml. Reaction Mixture	Pyruvate Conc. = 0.80 µM per ml. Reaction Mixture
400	0.031	0.059
410	0.035	0.068
420	0.046	0.087
430	0.071	0.128
435	0.148	0.259
437	0.234	0.421
439	0.314	0.622
441	0.342	0.675
443	0.348	0.695
445	0.343	0.681
447	0.334	0.669
450	0.317	0.663
460	0.245	0.493
480	0.118	0.238
500	0.045	0.093
520	0.016	0.032
540	0.004	0.009
560	0.001	0.003
580	0.000	0.001

TABLE III

The Relationship Between Absorbancy and Concentration of Pyruvic Acid Obtained by the Salicylaldehyde Method.

Concentration of Pyruvic Acid in µM per 1.5 ml. Reaction Mixture	Photometer Reading in KS Units
0.25	36
0.50	64
0.75	96
1.00	125
1.25	151
1.50	176
1.75	201
2.00	221

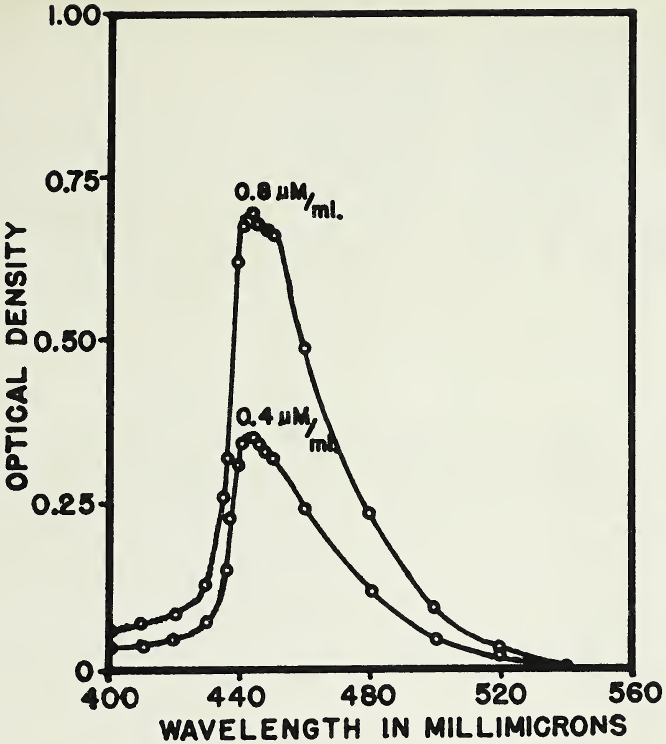

Fig. 3. The Relationship Between Absorbancy and Wavelength of Colored Solutions Obtained by the Salicylaldehyde Method for Pyruvic Acid.

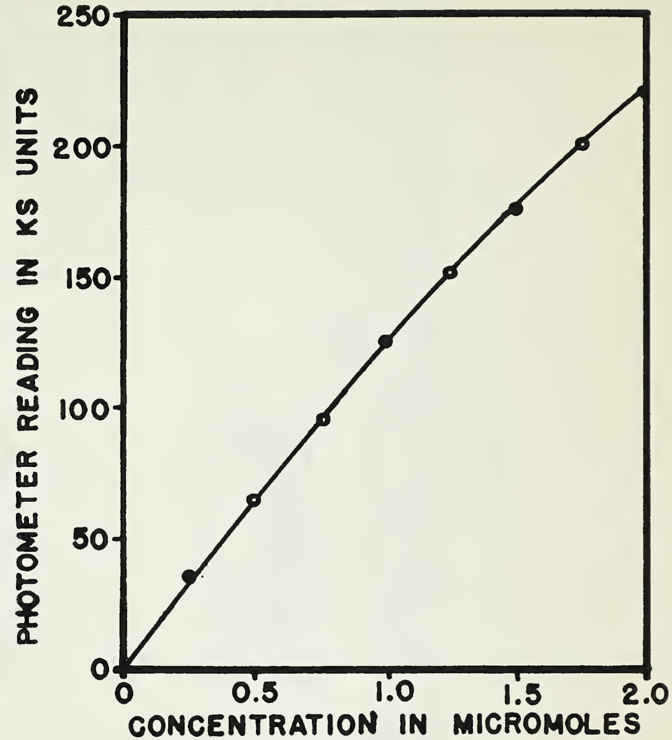

Fig. 4. The Relationship Between Absorbancy and Concentration of Pyruvic Acid Obtained by the Salicylaldehyde Method.

TABLE IV

The Relationship Between Absorbancy and Concentration of Aspartic Acid, Glutamic Acid, Alanine, Pyruvic Acid, Oxalacetic Acid and α-Ketoglutaric Acid Obtained by the Salicylaldehyde Method.

Amino or α-Keto Acid	Photometer Reading in KS Units 0.5 μM per 1.5 ml.	 1.0 μM per 1.5 ml.	 1.5 μM per 1.5 ml.
Aspartic	7	7	7
Glutamic	7	7	7
Alanine	8	7	7
Pyruvic	139	244	315
Oxalacetic	8	19	24
α-Ketoglutaric	9	10	13
Pyruvic in Presence of 90 μM Aspartic and 30 μM α-Ketoglutaric	64	125	176
Pyruvic in Presence of 90 μM Alanine and 30 μM α-Ketoglutaric	64	125	176

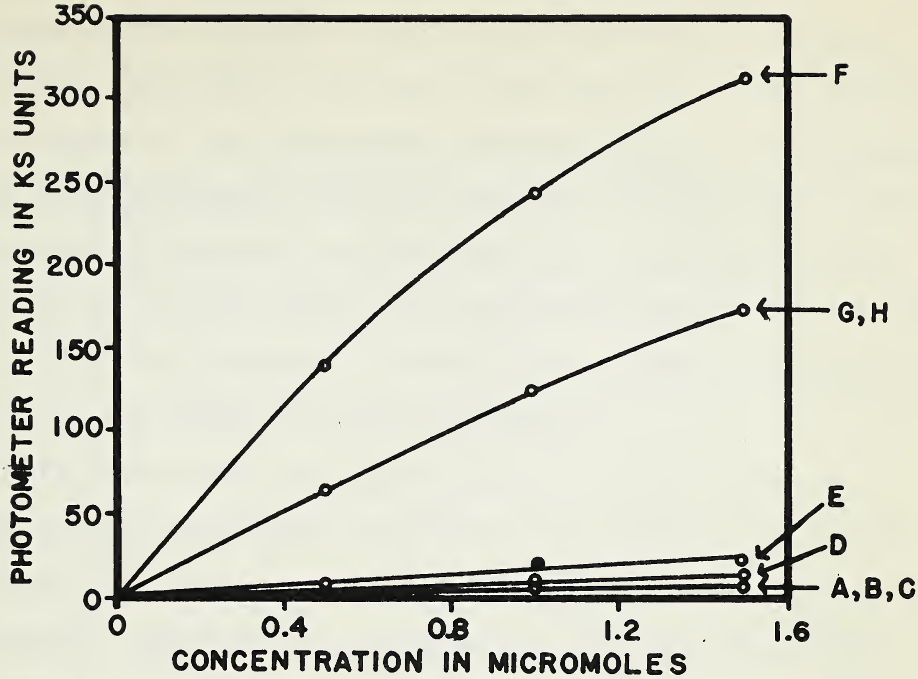

Fig. 5. The Relationship Between Absorbancy and Concentration of Aspartic Acid, Alanine, Glutamic Acid, Pyruvic Acid, Oxalacetic Acid and α-Ketoglutaric Acid, Obtained by the Salicylaldehyde Method.

Legend for Fig. 5.

A: Aspartic Acid.
B: Glutamic Acid.
C: Alanine.
D: α-Ketoglutaric Acid.
E: Oxalacetic Acid.
F: Pyruvic Acid.
G: Pyruvic Acid in Presence of 90 μM Aspartate and 30 μM α-Ketoglutarate.
H: Pyruvic Acid in Presence of 90 μM Alanine and 30 μM α-Ketoglutarate.

reactions react with salicylaldehyde to some extent. Pyruvic acid, however, is approximately twenty times more reactive than any of the other compounds which were tested. The reason for the depression of reactivity of pyruvic acid with salicylaldehyde upon the addition of 90 micromoles of aspartate or alanine and 30 micromoles of α-ketoglutarate per 1.5 ml. is not clear. It is possible that the decreased reactivity is a result of competition between substrate and pyruvate molecules for salicylaldehyde.

In order that the salicylaldehyde method could be used for the estimation of GOT, it was necessary to decarboxylate the oxalacetate which forms during the reaction to pyruvate. This was accomplished with the use of aniline citrate as described by Tonhazy *et al.* (140).

Optimum conditions for the transaminase assays

1. Source of enzymes

All experiments in this study were carried out on adult male albino rats which weighed approximately 250-300 grams. The intestinal tissue which was used in preliminary experiments for developing suitable assay conditions was obtained from non-fasting rats which were housed in group cages and maintained on Purina fox checkers and water *ad libitum*. The animals were killed by decapitation and the first 10 cm. of intestine from the pylorus was excised immediately and cleaned by rinsing the lumen three times with ice-cold

0.1 M phosphate buffer. After connective tissue, mesenteries and blood vessels were removed, the tissue was blotted gently with filter paper and weighed to the nearest hundredth of a gram. The cleaned intestinal tissue then was homogenized in cold 0.1 M phosphate buffer in an ice-cooled Potter-Elvehjem homogenizer (116) and the homogenate was diluted to 100 ml. with cold 0.1 M phosphate buffer. Fatty debris, which was usually suspended in the homogenate, was removed by centrifugation for five minutes at 1000 r.p.m. in an MSE clinical centrifuge, at room temperature. Two experiments were performed in order to determine whether any loss of transaminase activity occurred as a result of the centrifugation. In the first experiment, transaminase assays were carried out on centrifuged and uncentrifuged aliquots of the same homogenate. No difference in activity between the two samples was detected. This showed that centrifugation for five minutes at room temperature causes no detectable denaturation of transaminases and also that no appreciable amount of the enzymes is lost in the debris. In the second experiment, 5 ml. of homogenate was centrifuged and then the supernatant solution was decanted. The debris was washed once with 0.1 M phosphate buffer and tested for transaminase activity. The results from three repetitions of the experiment showed that debris from 5 ml. of homogenate contains no GPT activity and about 0.2 units of GOT activity.

As 0.2 ml. of homogenate is used in a transaminase assay, the loss of GOT activity which is caused by centrifugation of the homogenate would result in a difference of the final photometer reading of one KS unit and this is within the experimental error.

2. <u>Establishment of optimum conditions for enzyme assay</u>

Although glutamic-oxalacetic and glutamic-pyruvic transaminases of animal tissues have been determined at pH values varying from 7.3 to 8.0 (30, 37, 53, 56, 5, 7), most workers have used values at or close to pH 7.4. Preliminary experiments showed that in crude homogenates of rat intestine, optimal GOT activity occurs between pH 7.7 and 9.5, and optimum GPT activity occurs between pH 7.9 and 8.8. Consequently, a pH value of 8.5 was chosen for the initial studies. Although phosphate buffer is not always very efficient at pH 8.5, no change in the hydrogen ion concentration of the reaction mixture was found to occur during a sixty minute incubation period at 37°C.

The enzyme concentration in 0.2 ml. of homogenate per 1.5 ml. reaction mixture, and an incubation period of 30 minutes at 37°C. were chosen arbitrarily and then preliminary experiments were performed in order to determine the effect of substrate concentration. As a result of these experiments, a substrate concentration consisting of 90 micromoles aspartate and 30 micromoles α-ketoglutarate per

1.5 ml. reaction mixture was chosen for assaying glutamic-oxalacetic transaminase; similarly, a substrate concentration consisting of 90 micromoles alanine and 30 micromoles α-ketoglutarate per 1.5 ml. reaction mixture was chosen for assaying glutamic-pyruic transaminase. Under these conditions, approximately 2% of the α-ketoglutarate is transaminated during the reaction.

Next, the effect of enzyme concentration on transaminase activity was determined and the velocity of the transaminase reactions was found to be directly proportional to enzyme concentration for homogenate concentrations up to 0.5 ml. per 1.5 ml. reaction mixture. It was decided, therefore, that the use of the previously chosen enzyme concentration of 0.2 ml. homogenate per 1.5 ml. reaction mixture would be continued.

An experiment then was performed in order to determine the relationship between enzyme velocity and time of incubation. A linear relationship was shown for a period of 60 minutes, and hence, the use of an incubation period of 30 minutes at 37°C. was satisfactory.

Attention was turned next to a consideration of the effect of cofactors on the activity of intestinal glutamic-oxalacetic and glutamic-pyruvic transaminases. It now has been well established that pyridoxal phosphate is the coenzyme for transaminases (80, 53, 129, 130, 131, 6, 8, 96).

The coenzyme saturation curve for glutamic-oxalacetic transaminase has been worked out be O'Kane and Gunsalus (111). Using purified hog heart transaminase and a substrate system containing 45.5 micromoles α-ketoglutarate and 91 micromoles aspartate per ml. reaction mixture, they found that at a pH of 7.3 approximately 5 micrograms pyridoxal phosphate per ml. reaction mixture was required to saturate the enzyme. Kritzmann and Samarina (76) prepared a partially resolved preparation of glutamic-pyruvic transaminase from hog heart. They inactivated the enzyme by acidification and then showed that although reactivation could be effected by the addition of 1-5 micrograms pyridoxal phosphate, larger quantities (10-25 micrograms) failed to activate the enzyme. In our study, no increase in glutamic-oxalacetic or glutamic-pyruvic transaminase activity could be demonstrated upon addition of either 5 or 10 micrograms of pyridoxal phosphate. Patwardhan (114) has shown that ferrous iron may be involved in transamination of the glutamic-oxalacetic transaminase of green beans. We observed that addition of 5 micrograms per ml. reaction mixture of ferrous iron to incubation mixtures of intestinal glutamic-oxalacetic and glutamic-pyruvic transaminases resulted in no increase of activity. Evidence has been obtained recently by Happold and Turner (56) that purified glutamic-oxalacetic transaminase from sheep's heart muscle requires magnesium ions for optimum activity.

In the presence of phosphate buffer, however, magnesium ions precipitate as phosphate salts. Therefore, a study of the effect of magnesium ions on the activity of the transaminases in this investigation was not possible.

3. Unit of activity

The unit of glutamic-oxalacetic transaminase activity was defined arbitrarily, for purposes of this investigation, as the amount of enzyme which forms 1.0 micromole of oxalacetic acid under the described conditions. Similarly, the unit of glutamic-pyruvic transaminase activity was defined as the amount of enzyme which forms 1.0 micromole of pyruvic acid under the described conditions. Results are usually expressed as units of transaminase activity per gram of wet intestine.

4. Confirmation of the existence of GOT and GPT in rat intestine

The colorimetric estimation of glutamic-oxalacetic and glutamic-pyruvic transaminases by the salicylaldehyde method is based on the measurement of the α-keto acid which is formed by each transaminase system. Because the source of enzymes is a crude homogenate and because both transaminase reactions involve four components, measurement of one of these components would not provide conclusive evidence that glutamic-oxalacetic and glutamic-pyruvic transaminases actually exist in rat intestine. For this reason, the

following experiment was carried out.

A GOT reaction system was set up in each of two test tubes. The reaction in the first test tube was stopped at zero time by the addition of 8.5 ml. 95% ethyl alcohol, while the reaction in the second test tube was allowed to proceed for four hours at 37°C. and then it, also, was stopped by the addition of 8.5 ml. of 95% ethyl alcohol. The same procedure was carried out with the GPT system. The inactivated reaction mixtures were centrifuged for 10 minutes at 3000 r.p.m. in an MSE clinical centrifuge and 0.01 ml. of clear supernatant solution from each test tube was applied to Whatman No. 1 filter paper which previously had been washed in phosphate buffer, pH 12 (92). A solution containing 10 micrograms per ml. each of L-aspartate, L-glutamate and L-alanine also was applied to the same sheet of filter paper. The chromatogram then was run for 10 hours using phenol saturated with phsophate buffer, pH 12, as the solvent. On completion of the run it was dipped in diethyl ether and allowed to dry for four hours at room temperature and then the amino acid spots were developed by spraying the chromatogram with a solution of 0.5% w/v ninhydrin in 95% ethyl alcohol. A colored photograph of the chromatogram (Fig. 6) shows that glutamic acid is formed by both transaminase systems.

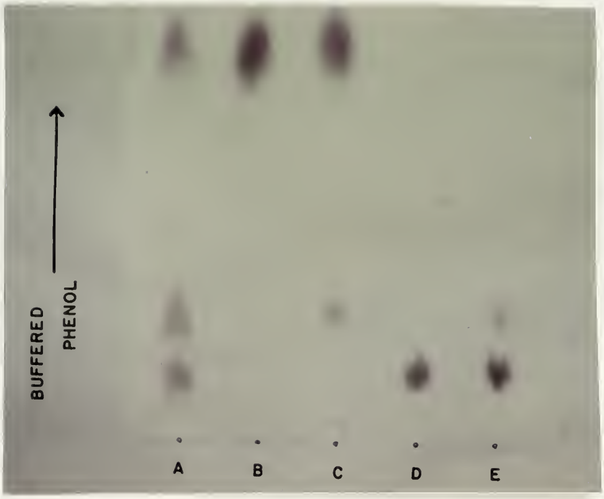

Fig. 6. Paper chromatogram showing the presence of glutamic-oxalacetic and glutamic-pyruvic transaminases in the small intestine of the rat. Complete GOT system: 90 µM L-aspartate, 30 µM α-ketoglutarate, 0.2 ml. homogenate made up to 1.5 ml. in 0.10 M phosphate buffer, pH 8.5. Complete GPT system: 90 µM L-alanine, 30 µM α-ketoglutarate, 0.2 ml. homogenate made up to 1.5 ml. in 0.10 M phosphate buffer, pH 8.5.

Legend for Fig. 6

A: Known amino acids. Lowest spot is aspartic acid; intermediate spot is glutamic acid; uppermost spot is alanine.
B: Complete GPT system; reaction stopped at zero time.
C: Complete GPT system; reaction stopped after four hours incubation at 37°C.
D: Complete GOT system; reaction stopped at zero time.
E: Complete GOT system; reaction stopped after four hours incubation at 37°C.

Summary

The procedure which was finally adopted for the assay of GOT and GPT was the following.

Approximately 0.5 grams (10 cm. section) of rat intestine was cleaned and homogenized in phosphate buffer and then the volume of the homogenate was adjusted to 100 ml. Fatty debris was removed from the homogenate by centrifuging for 5 minutes at 1000 r.p.m. and then 0.2 ml. of the supernatant solution was incubated with 1.3 ml. of substrate solution (see reagents) for 30 minutes at 37°C. The reaction was stopped by the addition of 0.5 ml. 10% trichloroacetic acid and one drop of aniline citrate was added to the GOT solutions in order to facilitate decarboxylation of oxalacetate to pyruvate. After the solutions had been allowed to stand at room temperature for twenty minutes, they were centrifuged, and one ml. of supernatant solution was transferred to a photometer tube. This was heated for 10 minutes at 37°C. with 1.0 ml. concentrated KOH solution and 0.3 ml. 2% salicylaldehyde reagent and then 3.0 ml. distilled water was added. The absorption then was determined immediately in a KS photometer using a KS-44 filter against a blank which was the same as the test solution except that the homogenate had been replaced with 0.1 M phosphate buffer.

<u>Precision and accuracy</u>

To establish the precision of the colorimetric transaminase method, six standard solutions containing 0.4 micromoles pyruvate per ml. reaction mixture were determined each day for a period of five days. The mean value of the photometer readings was found to be 78 ± 1.5*. Twenty eight of the values agreed within two KS units of the mean and two values agreed within 4 KS units of the mean. Similar precision was obtained when actual transaminase assays were being carried out.

Additions of 0, 0.5, 1.0, 1.5, and 2.0 micromoles pyruvic acid were made to incubation mixtures of both transaminase systems and then the pyruvic acid content in each mixture was determined. The average accuracy of four recovery experiments was found to be 1.5%.

4. Spectrophotometric Determination of Intestinal Glutamic-Oxalacetic Transaminase

The spectrophotometric method, which was used to verify some of the glutamic-oxalacetic transaminase kinetic data, was that of Nisonoff and coworkers (107, 108), as utilized by Cook (35).

In all experiments, the enzyme preparation was homogenate of rat intestine, as described previously. In order that aspartate and α-ketoglutarate could be added to the

* Standard deviation

reaction mixture one at a time, a separate solution of each was prepared. The reactions were carried out in silica absorption cells, and the reaction velocities were measured at a wavelength of 280 millimicrons in a Beckman DU spectrophotometer*. A temperature of 37°C. was maintained in the cell compartment with water circulated from a constant temperature bath through a thermospacer arrangement. The volume of the reaction mixture was always 3.0 ml.
The assay was carried out as follows.

Requisite amounts of buffer, enzyme preparation, and α-ketoglutarate solution were added to the absorption cell and the mixture was incubated at 37°C. for five minutes. At zero time, the aspartate solution (also at 37°C.) was added, and the cell was inverted six times and quickly returned to the cell compartment. Readings were started at 30 seconds and repeated every 15 seconds for a period of five minutes. The results then were plotted on graph paper and the increase in optical density per minute was determined.

At a wavelength of 280 millimicrons calibration curves were prepared for the substrates and products of the GOT system and they all were found to follow Beer's Law within the range of concentrations used in this study. Optical absorption coefficients, which were determined from the calibration curves, are shown in Table V. Nisonoff and

* Modified with Photovolt photomultiplier photometer Model 520M

coworkers (109) have shown that in their GOT systems a given concentration of any one of the components contributes to the optical density of a mixture of the components to the same degree that each would alone. This was verified with the substrates used in our studies. By means of the optical absorption coefficients in Table V, the change in optical density per minute was converted to velocity units expressed in micromoles per ml. per minute. The production of one micromole per ml. each of oxalacetic acid and glutamic acid causes an increase in optical density equal to (0.538 + 0.0008) - (0.0215 + 0.0002) = 0.5176. Therefore, the velocity in optical density units per minute divided by 0.5176 gives the velocity in micromoles per ml. per minute.

Representative curves showing the time course of the reaction of four different incubation mixtures are presented in Fig. 7.

TABLE V

Optical Absorption Coefficients of Glutamic-Oxalacetic Transaminase Substrates and Products at a Wavelength of 280 Millimicrons.

Substrate or Product	Optical Absorption Coefficient, Optical Density Unit per µM per ml.
Oxalacetate	0.538
Alpha-Ketoglutarate	0.0215
Aspartate	0.0002
Glutamate	0.0008

Concentration range of oxalacetate: 0 to 3 µM per ml.
Concentration range of other substrates: 0 to 50 µM per ml.
All substrates dissolved in 0.10 M phosphate buffer, pH 8.5.

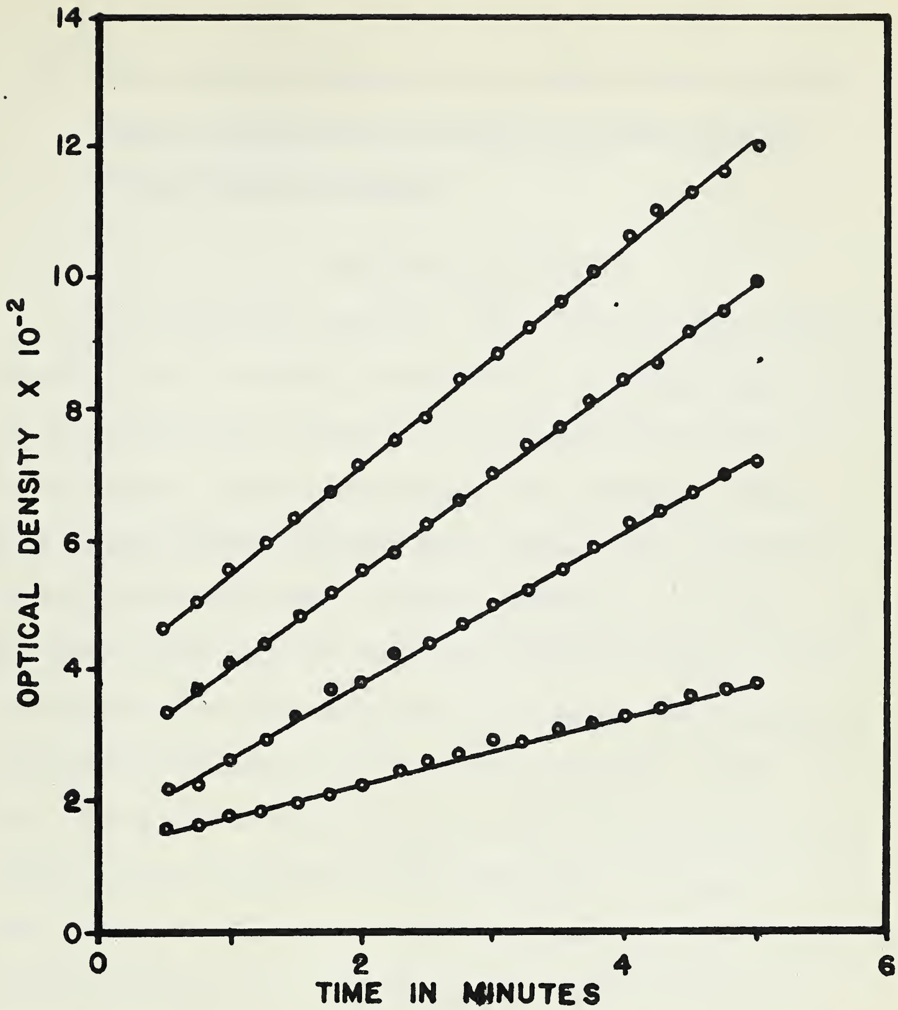

Fig. 7. Typical time course curves of the glutamic-oxalacetic transaminase reaction at varying pH values. Tangents show initial velocities. Wave-length, 280 mμ. Complete reaction system: 0.5 ml. enzyme preparation; 50 μM L-aspartate; 50 μM α-ketoglutarate; 0.10 M phosphate buffer at varying pH values, to make final volume of 3.0 ml. Temperature, 37°C.

B. <u>The Determination of Free Amino Acids in the Small Intestine of the Rat by Quantitative Paper Chromatography</u>.

1. Preliminary Studies

In the initial studies, the first 10 cm. section of intestine was excised, immediately cleaned, then homogenized in cold 80% ethyl alcohol in an ice-cooled Potter-Elvehjem homogenizer. After centrifugation, varying amounts of the clear supernatant extract were applied to one corner of sheets of Whatman No. 1 filter paper (8 X 8 in.). The chromatograms were placed in a rectangular chromatography chamber and run for approximately seven hours using phenol saturated with water as the first solvent. After they had been dried at room temperature, the chromatograms were placed in the second solvent (60 parts propanol, 30 parts conc. ammonia, 10 parts water) in such a manner that the solvent migrated at a right angle to the direction of ascension of the first solvent. The chromatograms again were allowed to run for approximately seven hours; then, they were removed and allowed to dry at room temperature. The amino acid spots were developed by spraying the chromatograms with 0.5% w/v ninhydrin in 95% ethyl alcohol. As a

result of chromatographing several intestinal extracts, it was found that the amino acids which are present in rat intestine in sufficient quantity to permit their quantitative estimation are aspartic acid, glutamic acid, glycine, and alanine. The method which was finally adopted for the separation of these four amino acids was that of McFarren (92), which makes use of buffered filter paper and buffered phenol in order to achieve adequate separation of amino acids.

2. Reagents

1. 0.034 M Phosphate buffer, pH 12.0, (92)

4.757 grams Na_2HPO_4 and 1.38 grams NaOH were dissolved in water and then the volume was adjusted to 1000 ml.

Na_2HPO_4: Anhydrous, Fisher Certified Reagent.

NaOH: Merck and Co. Ltd.

2. 80% v/v Ethyl alcohol
3. 72% v/v Ethyl alcohol
4. 0.5% w/v Ninhydrin in 95% ethyl alcohol

Ninhydrin: Dougherty Chemicals.

3. Extraction and Chromatographic Separation of Intestinal Amino Acids.

Chromatography paper was prepared by washing sheets of Whatman No. 1 filter paper (7 X 15 in.) in phosphate buffer, pH 12.0, which then were dried at room temperature.

The amino acid extracts were prepared by cleaning approximately 0.5 grams (10 cm. section) of rat intestine, homogenizing in cold 80% ethyl alcohol (total volume of homogenate, 2.0 ml.) in an ice-cooled Potter-Elvehjem homogenizer, and then centrifuging the homogenate for five minutes at 3000 r.p.m. in an MSE clinical centrifuge. A pencil line was drawn, parallel to the 15 in. end and 3 in. from the edge, on each sheet of filter paper. The extraction mixtures were applied to the pencil line, as single spots, with a 10 µl. pipette. In order to accumulate 100 µl. of extract on each spot, the application with the 10 µl. pipette was repeated 10 times, by allowing the fluid to dry after each application. The papers then were placed in a cylindrical chromatography tank which was suitable for one-dimensional descending chromatography. The solvent was equilibrated in a separatory funnel by shaking phenol with phosphate buffer, pH 12.0. When the layers had separated, the solvent-rich layer was placed in the trough and the buffer-rich layer was placed in the bottom of the chamber. The chromatograms were allowed to run at room temperature for approximately 10 hours; then they were dipped in diethyl ether three times, suspended by one end, and air-dried. Color on the amino acid spots was developed by first spraying with 0.5% ninhydrin reagent and then drying the chromatograms for 10 minutes at 70°C. Copies of two typical chromatograms are shown in Fig. 8. Each time an aliquot of

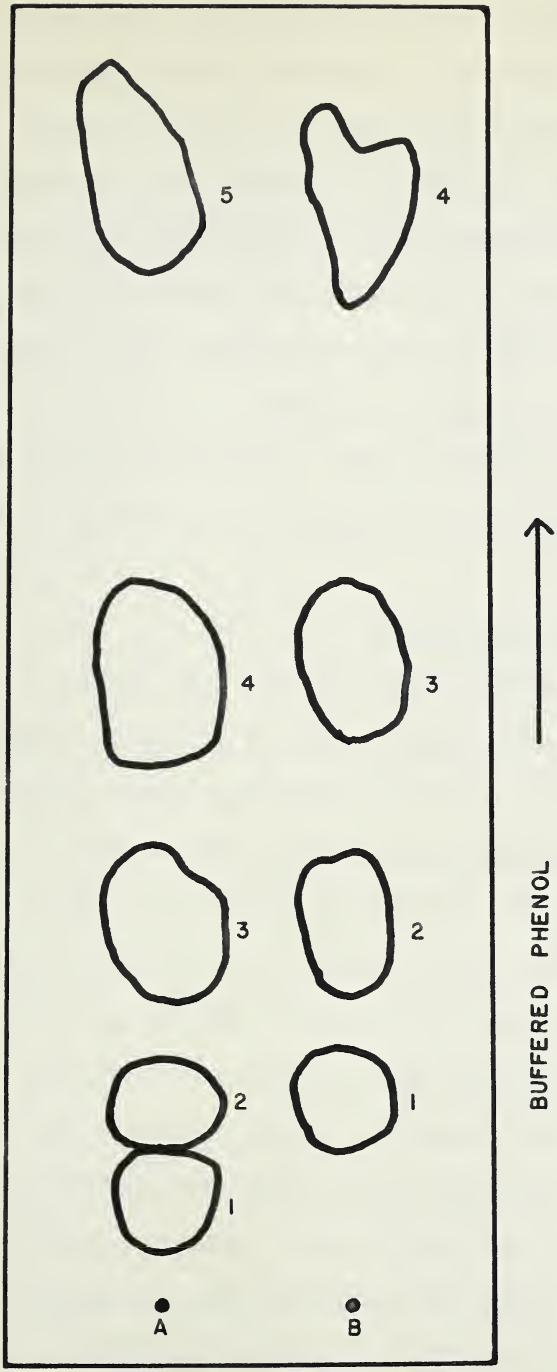

Fig. 8. Copies of typical buffered chromatograms. Conditions: phenol saturated with 12.0 pH buffer, paper buffered at pH 12.0.

A: 100 μl. intestinal extract.

1. Unknown substance
2. Aspartic acid
3. Glutamic acid
4. Glycine
5. Alanine

B: Known mixture of amino acids.

1. Aspartic acid
2. Glutamic acid
3. Glycine
4. Alanine

intestinal extract was chromatographed, an unknown ninhydrin-reacting substance appeared in position 1, as demonstrated in Fig. 8. The low R_f value of this substance suggests the presence of several ionized groups on the molecule, and thus, it was suspected that this unknown compound could be a di- or tri-peptide. While this thesis was in the process of being written, Tuba and Neufeld (143) showed that glutathione possessed the same R_f value as the unknown substance. Thus, pending further verification, the compound was assumed to be glutathione.

4. Quantification

After the chromatograms had been developed, they were left overnight at room temperature. Quantitative estimation of the amino acids was carried out the next day as follows.

The chromatograms were held up to a strong light and a circle was drawn around each amino acid spot. Each spot then was cut out, shredded, and placed in a 15 ml. test tube. Four ml. of 72% ethyl alcohol was added, and then the tubes were stoppered tightly and vigorously shaken for 60 minutes in an electric shaker. After being shaken, the tubes were centrifuged at 3000 r.p.m. for five minutes. The ethyl alcohol solution elutes the color and the centrifugation sediments paper particles present in the solution. By use of a Beckman DU spectrophotometer, equipped with matched

cells of 10-mm. light path, the absorbancy of the eluates was determined against a blank of 72% ethyl alcohol. Calibration curves, relating absorbancy of ninhydrin color to micrograms of amino acid applied to the paper, were determined with each set of chromatograms. The quantities of amino acids in the intestinal extracts then were determined from these standard curves.

Absorption spectra were determined on eluates of each of the four amino acids after they had been subjected to the ninhydrin reaction (Figs. 9, 10, 11, 12). It was found that highest sensitivity and maximum absorption of aspartate, glutamate, glycine, and alanine occur at wavelengths of 595, 575, 450, and 575 millimicrons. Consequently, subsequent absorption measurements of eluates of ninhydrin-reaction products of these amino acids were determined at the above-mentioned wavelengths.

Typical calibration curves of ninhydrin-reaction products of each of the amino acids are shown in Fig. 13. Although a white piece of chromatogram of the same size as the amino acid spots was not included in the blank solution, the curves pass through the origin.

5. Precision and Accuracy

As the color intensity of the spots is dependent on humidity and room temperature, calibration curves were not

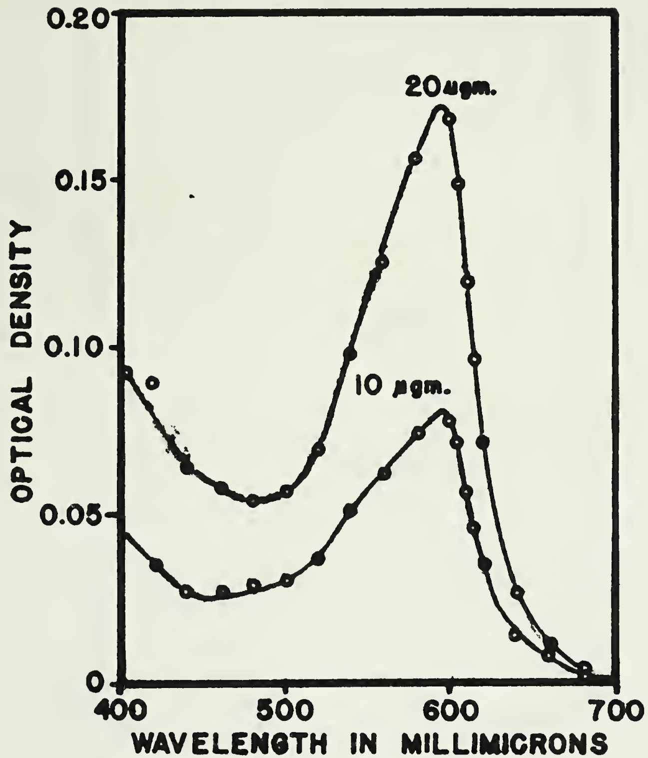

Fig. 9. The relationship between absorbancy and wavelength for colored eluates of ninhydrin-reaction products of aspartic acid.

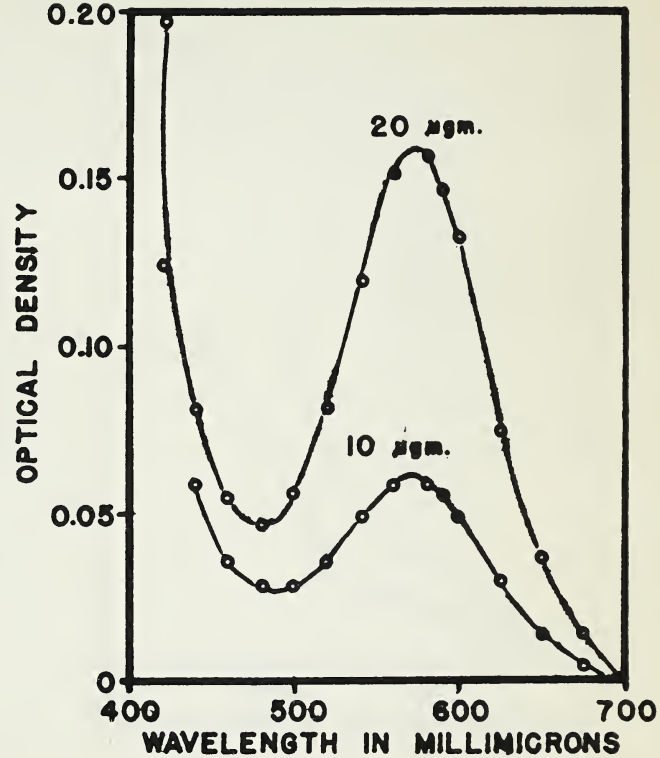

Fig. 10. The relationship between absorbancy and wavelength for colored eluates of ninhydrin-reaction products of alanine.

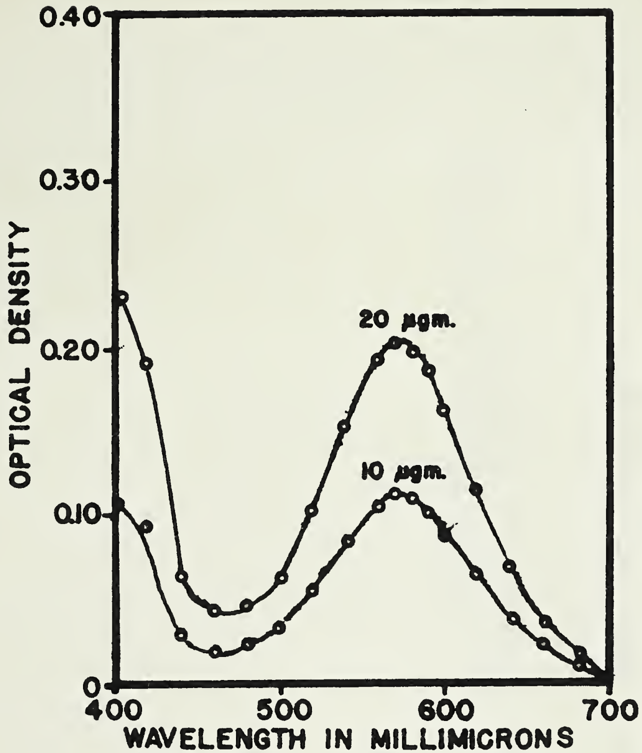

Fig. 11. The relationship between absorbancy and wavelength of colored eluates of ninhydrin-reaction products of glutamic acid.

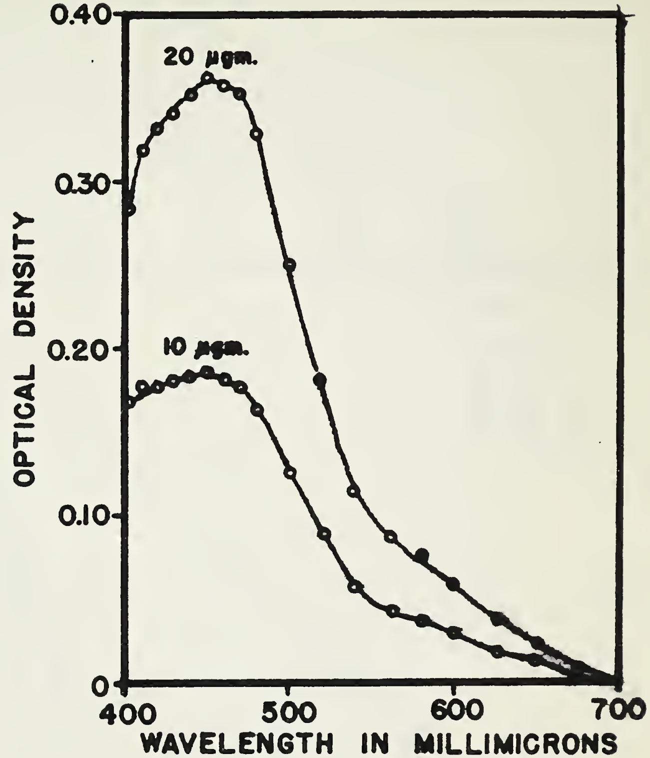

Fig. 12. The relationship between absorbancy and wavelength of colored eluates of ninhydrin-reaction products of glycine.

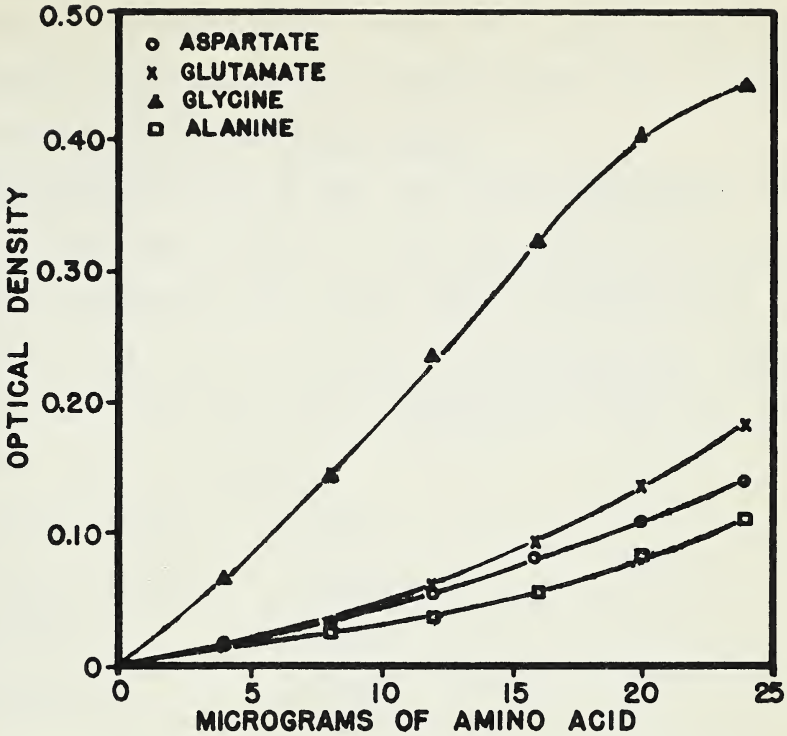

Fig. 13. Absorbancy of ninhydrin-reaction products as a function of the amount of compound applied to the chromatogram.

reproducible from day to day. Replicates run on the same day, however, usually agreed within 2% transmittancy units. Occasionally, when absorbancy readings were high, duplicates agreed within 3% transmittancy units.

Additions of 0, 4, 8, 12, 16, and 20 micrograms of each amino acid were made to 2 ml. aliquots of an intestinal homogenate preparation and then, after centrifugation, the amino acid content was estimated in each mixture. Average accuracies obtained from four repetitions of the recovery experiment were as follows:

Aspartic acid -- + 6.5%

Glutamic acid -- + 10.0%

Glycine -- - 7.9%

Alanine -- - 2.0%.

CHAPTER III

THE DISTRIBUTION OF TRANSAMINASE ACTIVITY IN THE SMALL INTESTINE OF THE RAT

THE DISTRIBUTION OF TRANSAMINASE ACTIVITY IN THE SMALL INTESTINE OF THE RAT

1. Introduction

Indirect evidence recently has been reported for the exostence of transaminases in the small intestine of cats, rabbits, dogs, and rats. Direct quantitative estimations and distribution studies of transaminases in the small intestine of mammals apparently have not been carried out.

Matthews and Wiseman (90), by making use of the technique of suspending a section of intestine in oxygenated saline, were probably the first workers to demonstrate the existence of transaminases in rat intestine. They found that one hour after the introduction of glutamic or aspartic acid into the solution in the lumen of the intestinal preparation, the serosal fluid contained both dicarboxylic acid and alanine. Neame and Wiseman (104), using an *in vivo* technique, showed that when glutamic or aspartic acid is absorbed from the small intestine of the dog, an increased concentration of alanine occurs in the mesenteric venous blood. In the following year, these same workers (105) produced evidence that transamination also occurs in the small intestine of cats and rabbits.

In order to establish the amount and localization of GOT and GPT activity in the small intestine of the rat, the following experiments were carried out by us.

2. Experimental

The experimental animals used in the following study were adult male albino rats weighing approximately 250-320 grams. They were housed in individual cages and maintained on an <u>ad libitum</u> diet of Purina fox checkers and water.

After the animal had been killed by decapitation, the entire small intestine was excised and then, starting from the pyloric end, it was cut into 10 cm.-long segments. Following this, each section was cleaned immediately by rinsing with cold 0.10 M phosphate buffer and by removing mesenteries and blood vessels. The sections then were carefully blotted dry with filter paper and weighed to the nearest hundredth of a gram. Finally, the cleaned intestinal sections were homogenized in cold phosphate buffer in an ice-cooled Potter-Elvehjem homogenizer, and the homogenate and washings were made up to volume in a 100 ml. volumetric flask. Using the colorimetric procedure described previously, transaminase assays then were performed on 0.2 ml. aliquots of the homogenate.

3. Results and Discussion

The results of the experiment, which are presented in Tables VI and VII, corroborate the findings of Matthews and Wiseman (90), and provide further evidence that transaminases exist in the small intestine of the rat. Unlike the distribution in rat intestine of sucrase (9) and alkaline phosphatase (141), which are concentrated almost entirely in the duodenum and jejunum, transaminase activity is apportioned approximately evenly along nearly the entire length of the small intestine. Fig. 14 and 15 clearly demonstrate that there is a significant decline of transaminase activity only in the terminal thirty cm. of the intestine and that this decrease is much greater for the glutamic-pyruvic than the glutamic-oxalacetic transaminase. Although no highly significant difference was found between the transaminase activities of sections 1 to 8, Fig. 15 shows that GPT activity appears to be greatest in sections 3, 4, 5, and 6.

Inasmuch as future studies were to involve the simultaneous estimation of transaminase activity and free amino acid levels, it seemed appropriate that these determinations should be carried out on adjacent 10 cm. segments of the intestine. Earlier, when the chromatographic procedure for estimation of amino acids in intestine was being developed, it was established that the first two 10 cm.

TABLE VI

The Distribution of GOT Activity in the Small Intestine of the Adult Male Albino Rat

Section Number	Number of Animals	GOT Activity (Units per gram of wet tissue)
1	5	810 ± 17*
2	5	824 ± 16
3	5	809 ± 12
4	5	836 ± 4
5	5	812 ± 18
6	5	769 ± 38
7	5	788 ± 11
8	5	788 ± 18
9	5	749 ± 20
10	5	725 ± 23
11	5	701 ± 26
12	5	698 ± 22

* Standard error of the mean.

TABLE VII

The Distribution of GPT Activity in the Small Intestine of the Adult Male Albino Rat

Section Number	Number of Animals	GPT Activity (Units per gram of wet tissue)
1	5	842 ± 38*
2	5	889 ± 37
3	5	905 ± 44
4	5	923 ± 39
5	5	933 ± 28
6	5	918 ± 48
7	5	869 ± 25
8	5	838 ± 31
9	5	797 ± 41
10	5	578 ± 37
11	5	396 ± 32
12	5	350 ± 43

* Standard error of the mean.

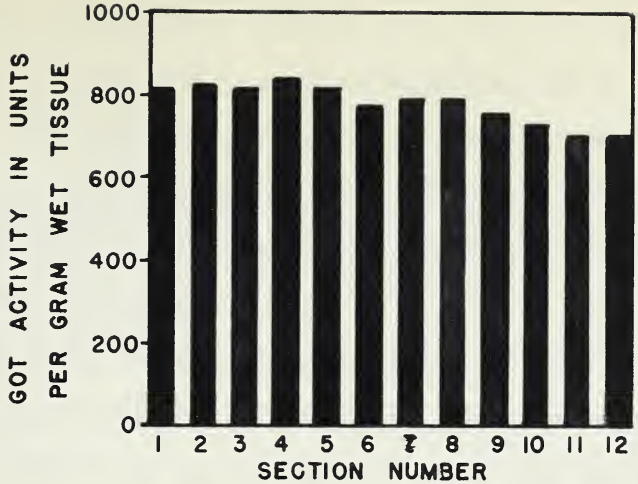

FIG. 14. THE DISTRIBUTION OF GOT ACTIVITY IN THE SMALL INTESTINE OF THE ADULT MALE ALBINO RAT.

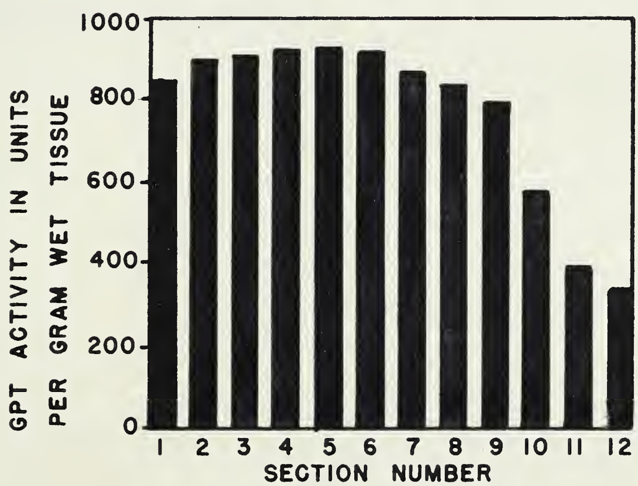

FIG. 15. THE DISTRIBUTION OF GPT ACTIVITY IN THE SMALL INTESTINE OF THE ADULT MALE ALBINO RAT.

sections contain the highest concentrations of free amino acids. Consequently, as a compromise, it was decided that in the following studies, the second 10 cemtimeters of intestine would be used for amino acid estimations, and transaminase assays would be carried out on the third one-decimeter section.

In order to ascertain how much transaminase activity is removed from the intestine by the washing process, assays were carried out on rinsings from the third 10 cm. segment of four rats. While no GOT activity could be detected, an average GPT activity of approximately 20 units was found in the washings of each 10 cm. section. As this constitutes approximately 2% of the total GPT activity in the section, the loss was disregarded.

CHAPTER IV

KINETIC STUDIES OF RAT INTESTINAL GLUTAMIC-OXALACETIC AND GLUTAMIC-PYRUVIC TRANSAMINASES

A. Introduction

Studies of enzyme reaction kinetics lead to a knowledge of the optimum conditions under which the enzyme should be assayed and the results of such an investigation also provide information on the mechanism of the enzyme reaction. With this in mind, a study of the kinetics of glutamic-oxalacetic and glutamic-pyruvic transaminases in rat intestine was undertaken and the following factors were investigated:

1. Hydrogen-ion concentration
2. Substrate concentration
3. Temperature
4. Time of reaction
5. Enzyme concentration
6. Storage time.

In all cases, the enzyme preparations were homogenates of the third 10 cm. section of the small intestine of non-fasting rats. The homogenates were prepared as described previously.

B. Effect of Hydrogen-Ion Concentration

1. Experiments and Results

Although reports of pH optima for transaminase activity in animal tissues give values which are at or close to 7.4 (30, 53, 37, 107), preliminary studies in our laboratory

showed that the pH optima of intestinal GOT and GPT are at 8.5. In order to confirm these preliminary studies, the effect of pH on the rate of intestinal GOT and GPT was determined more thoroughly.

The results of the experiment are presented in Table VIII and are represented graphically in Figs. 16 and 17. A complete reaction system contained 90 micromoles of the appropriate amino acid, 30 micromoles α-ketoglutarate, and 0.2 ml. of homogenate made up to 1.5 ml. in 0.10 M phosphate buffer. The pH of each reaction mixture was adjusted to the desired value by regulating the acid:base ratio of the phosphate salts. No change of pH was found to occur in any of the reaction mixtures during the incubation period. All pH measurements, which were controlled to within $\pm$ 0.02 units, were carried out with a Beckman Model G pH meter. The results, which confirm the preliminary studies, show that optimum GOT and GPT activities occur at a pH of 8.5. However, the fact that the pH optima of the two intestinal transaminases were found to be so different from the optimal values of tissue transaminases obtained by other workers was disturbing. Consequently, to test the validity of the data obtained by the colorimetric method, the experiment was repeated using the spectrophotometric procedure which is described in chapter II.

TABLE VIII

The Effect of pH on the Rate of Intestinal GOT and GPT Activity of the Rat. Complete GOT System: 90 µM L-aspartate, 30 µM α-ketoglutarate, 0.2 ml. homogenate made up to 1.5 ml. in 0.10 M phosphate buffer. Complete GPT System: 90 µM L-alanine, 30 µM α-ketoglutarate, 0.2 ml. homogenate made up to 1.5 ml. in 0.10 M phosphate buffer. Incubation time, 30 min. Temperature, 37°C.

pH	GOT Activity in Units per Gram of Wet Tissue	pH	GPT Activity in Units per Gram of Wet Tissue
2.72	32	2.14	8
4.22	56	3.24	24
5.82	468	4.69	32
6.67	643	5.79	389
6.97	651	7.00	627
7.18	682	7.20	677
7.27	682	7.27	690
7.33	698	7.37	690
7.42	722	7.47	730
7.52	715	7.48	738
7.66	715	7.69	730
7.79	736	7.83	738
8.03	738	8.11	770
8.36	746	8.43	770
8.67	738	8.73	762
9.00	736	9.12	730
9.46	722	9.59	564
10.29	643	10.43	56
11.48	56	10.79	32
		11.25	16

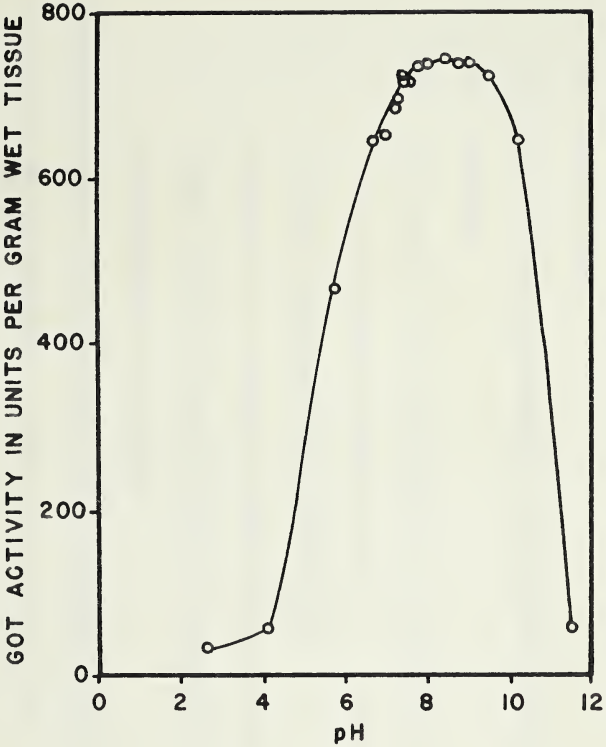

Fig. 16. The effect of pH on the rate of intestinal GOT activity of the rat. Complete GOT system: 90 µM L-aspartate, 30 µM α-ketoglutarate, 0.20 ml. homogenate made up to 1.5 ml. in 0.10 M phosphate buffer. Incubation time, 30 min. Temperature, 37°C.

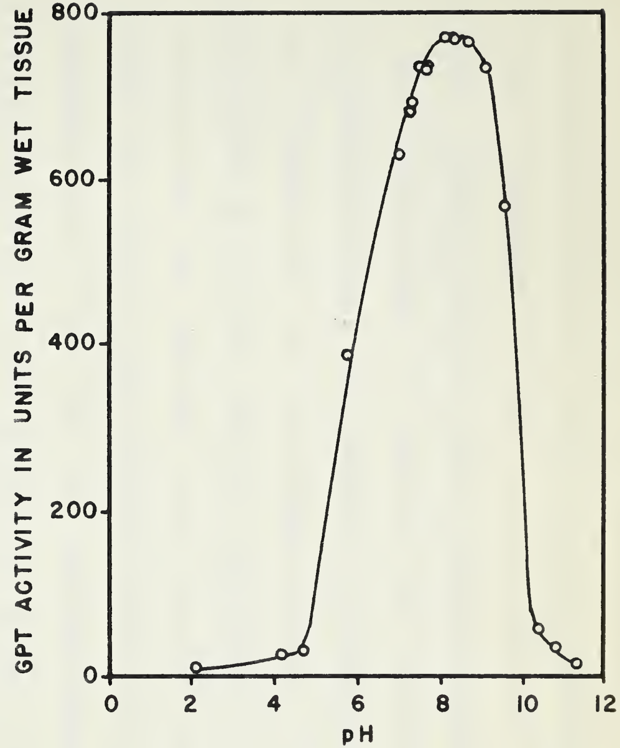

Fig. 17. The effect of pH on the rate of intestinal GPT activity of the rat. Complete GPT system: 90 µM L-alanine, 30 µM α-ketoglutarate, 0.20 ml. homogenate made up to 1.5 ml. in 0.10 M phosphate buffer. Incubation time, 30 min. Temperature, 37°C.

In this experiment, the reaction system contained 150 micromoles each of L-aspartate and α-ketoglutarate, and 0.5 ml. of homogenate made up to 3.0 ml. in 0.10 M phosphate buffer. Again, the pH of each reaction mixture was adjusted to the desired value by regulating the acid:base ratio of the phosphate salts. The results are presented in Table IX. Fig. 18 shows that the bell-shaped pH curve obtained by measurement of initial reaction velocities is considerably narrower than that obtained using the colorimetric method. Whereas the optimal range obtained by the salicylaldehyde procedure is pH 7.8 to 9.5, the spectrophotometric method produces an optimal range of pH 8.5 to 9.0. The use of either method, however, leads to the conclusion that maximum activity of intestinal transaminases occurs at pH 8.5 - 8.8, and not at pH 7.4.

In the above experiments, phosphate buffer was employed for two reasons:

1. At first, the pH optima of intestinal transaminases were expected to be at pH 7.4. Phosphate salts buffer efficiently at this pH.
2. Nisonoff and Barnes (107) had provided evidence that phosphate ions exert a catalytic effect on transaminase activity.

However, upon the discovery that optimal activity of intestinal transaminases occurs at a pH at which phosphate

salts do not buffer efficiently, a different buffer system was chosen and, using the spectrophotometric procedure, the effect of hydrogen-ion concentration on intestinal GOT was determined again.

Barbital buffer, because it works well at pH 8.5, was chosen for the experiment. The conditions of each reaction mixture were as follows:

substrate concentration - 150 micromoles each of L-aspartate and α-ketoglutarate.

enzyme concentration - 0.25 ml. homogenate

buffer concentration - 0.05 M

total volume - 3.0 ml.

temperature - 37°C.

The pH of each reaction mixture was adjusted to the desired value by regulating the ratio of barbital to sodium barbital. As shown in Table X and Fig. 19, the pH curve obtained by using barbital buffer demonstrates that a deviation of 0.12 units from the pH optimum of 9.08 results in a considerable loss (approximately 25%) of enzyme activity. This being the case, barbital would not provide a satisfactory buffer system for the routine assay of intestinal transaminases. Consequently, on the basis that no change in hydrogen-ion concentration had been found to occur when reaction mixtures were buffered with phosphate salts (chapter II), phosphate buffer, even though it is not always

TABLE IX

The Effect of pH on the Rate of Intestinal GOT Activity of the Rat. Complete GOT System: 150 μM L-aspartate, 150 μM α-ketoglutarate, 0.5 ml. homogenate made up to 3.0 ml. in 0.10 M phosphate buffer. Temperature, 37°C.

pH	Initial Reaction Velocity, μM Oxalacetate Formed per ml. per min. ($\times 10^{-2}$)
6.81	5.8
7.09	6.0
7.36	7.2
7.72	8.1
7.85	7.8
7.94	8.9
8.32	11.2
8.47	14.3
8.77	15.3
8.94	14.6
9.00	14.8
9.18	13.2
9.32	9.3
9.33	9.5
9.54	6.8
10.08	2.9

TABLE X

The Effect of pH on the Rate of Intestinal GOT Activity of the Rat. Complete GOT System: 150 µM L-aspartate, 150 µM α-ketoglutarate, 0.25 ml. homogenate made up to 3.0 ml. in 0.05 M barbital buffer. Temperature, 37°C.

pH	Initial Reaction Velocity, µM Oxalacetate Formed per ml. per min. (X 10^{-2})
7.29	3.7
7.52	4.2
8.02	4.6
8.42	5.2
8.77	5.4
8.83	5.5
8.96	5.8
9.07	6.1
9.08	6.2
9.20	4.7
9.30	3.3
9.52	2.3
9.73	1.3
9.76	1.1
9.94	1.0

TABLE [illegible]

The Effect of pH on the Rate of [illegible] [illegible] [illegible] of the Rat. [illegible] [illegible] [illegible] [illegible] 150 [illegible] [illegible], [illegible] ml. [illegible] [illegible] ml. to 0.05 M [illegible] buffer. Temperature, [illegible].

Initial [illegible] [illegible] [illegible] (ml. per min. [illegible])	pH
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
1.0	[illegible]

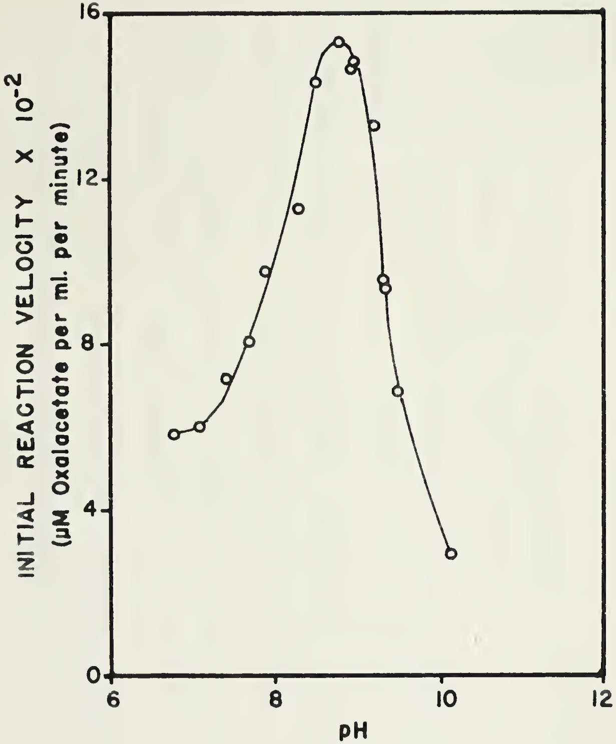

Fig. 18. The effect of pH on the rate of intestinal GOT activity of the rat. Complete GOT system: 150 µM L-aspartate, 150 µM α-ketoglutarate, 0.50 ml. homogenate made up to 3.0 ml. in 0.10 M phosphate buffer. Temperature, 37°C.

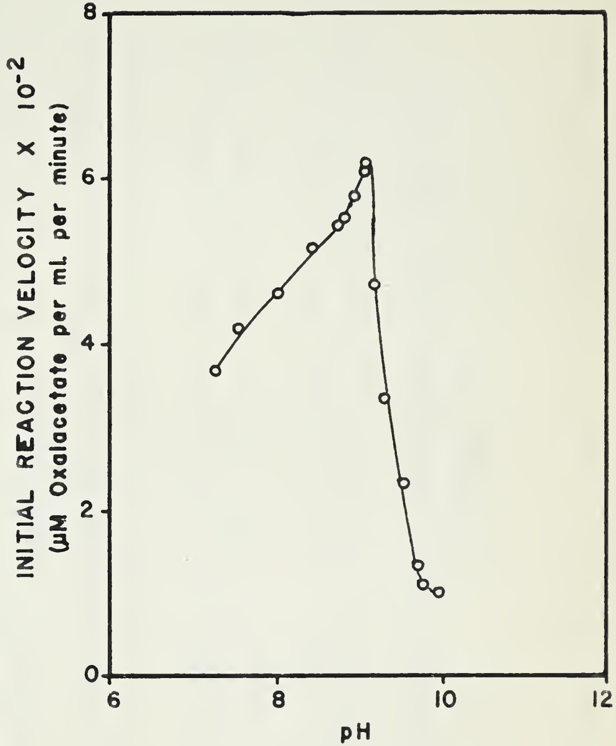

Fig. 19. The effect of pH on the rate of intestinal GOT activity of the rat. Complete GOT system: 150 µM L-aspartate, 150 µM α-ketoglutarate, 0.25 ml. homogenate made up to 3.0 ml. in 0.05 M barbital buffer. Temperature, 37°C.

efficient at pH 8.5, was finally chosen for use in the routine estimation of intestinal transaminases.

2. Discussion

The data of the above experiments provide no simple means for explaining the reason why a pH optimum of 8.5 was obtained in our laboratory while other workers found animal transaminases to work best at pH 7.4. Possibly the explanation lies in the fact that many factors effect the pH optimum of enzymes. For example, a crude intestinal homogenate was employed in our studies while most of the reported kinetic data were obtained from experiments which were carried out with either partially or highly purified enzyme preparations. Consequently, in our studies such factors as:

1. Concentration of cofactors
2. Presence of other enzymes
3. Concentration of activators and inhibitors

were not controlled. Studies of the effect of pH on transaminase activity are further complicated because the enzyme reaction involves four ionizable substrates. The concentration of the substrates, which is dependent on the concentration of the enzyme, would also effect the pH optimum of the reaction. Despite these uncertainties, however, our studies did demonstrate two important properties of

intestinal transaminases.

1. The bell-shaped pH curve obtained by using the spectrophotometric method was much narrower than that obtained using the colorimetric procedure. This indicates that the longer incubation time of the latter method allows some denaturation of the enzyme to occur.
2. The type of buffer employed was shown to affect the pH optimum considerably. Whereas use of phosphate buffer resulted in bell-shaped pH curves, use of barbital buffer produced a spear-shaped pH curve with a pH optimum about 0.5 pH units higher than that obtained with phosphate buffer.

Although phosphate buffer was found to be satisfactory for use in the routine assay of intestinal transaminases, it would be quite undesirable for further investigations involving the effect of pH. As the pH is varied from acidic to basic values, the ratio of the concentrations of the two phosphate ions changes and it would be expected that the effects of binding of the mono- and di-valent ions to the enzyme would be quite different. Tris-(hydroxy)-amino methane buffers probably would prove useful for further studies of this nature.

Finally, it is worth noting that Tuba and Harker (142) have determined the effect of pH on intestinal transaminases of C3H mice. Using the salicylaldehyde assay procedure

and phosphate buffer, they obtained pH curves for GOT and GPT which are almost identical with those obtained in the present investigation.

C. The Effect of Substrate Concentration

1. Introduction

The study of the effect of substrate concentration on transamination is complicated by the presence of two substrates in the reaction system. Consequently, in order to explain the results of such a study, the classical theory of Michaelis and Menten (101), which takes into consideration a change in one substrate only, must be extended to take into account a ternary complex consisting of the enzyme molecule and two substrate molecules.

The only previously published accounts which attempt to formulate and test equations which deal with the effect of substrate concentration on transamination apparently have been those of Nisonoff and Barnes (107) and Cook (35). The mathematical treatment proposed by Nisonoff and Barnes (107), who worked with pig heart GOT, is an extension of that introduced by Van Slyke and Cullen (146) for a one-substrate enzyme system. This approach differs from that of Michaelis and Menten (101) in that it assumes irreversible combination of enzyme with substrate. It is well known, however, that the glutamic-oxalacetic transaminase reaction is reversible. The mathematical treatment proposed by Cook (35), on the other hand, is an extension of the theory

of Michaelis and Menten (101), and it does assume that enzyme and substrate combine reversibly. The source of glutamic-oxalacetic transaminase in Cook's studies was corn radicles.

For the present studies, the reaction mechanism used by Cook was adopted.

2. Mathematical Treatment

For the sake of brevity, Cook (35), in his publication of the mathematical treatment of the relation of reaction rate to substrate concentration for GOT, presented only key equations. In the following text, these key equations have been repeated. However, in order to show the derivation of these equations, the intervening mathematical manipulations have been worked out and included.

The adopted reaction mechanism is based on the following assumptions:

1. That the two substrates occupy different sites on the enzyme, and that it is not necessary for one combination to occur before the other can take place.
2. That the enzyme-substrate complexes dissociate, and that each substrate is free to combine with, and dissociate from, its specific locus without being influenced by the other.
3. That the rate of breakdown of the ternary compound to free enzyme is small, and does not appreciably affect

the equilibrium constants of the other reactions. This assumption was made by Michaelis and Menten, but criticized by Briggs and Haldane (17). If this assumption does not hold, Km is not a true equilibrium constant, but the analysis of the equations for the single enzyme system is not influenced.

The separate enzyme-substrate combination reactions can then be represented:

$$E + A \rightleftharpoons EA$$

$$E + B \rightleftharpoons EB$$

$$EB + A \rightleftharpoons EAB$$

$$EA + B \rightleftharpoons EAB$$

$$EAB \longrightarrow E + \text{products}$$

in which,

A and B are the substrates, E = free enzyme, and E_t is equal to the total enzyme.

The dissociation constants, or Km's, can then be written,

$$Km1 = \frac{(E)(A)}{(EA)} = Km'1 = \frac{(EB)(A)}{(EAB)}$$

$$Km2 = \frac{(E)(B)}{(EB)} = Km'2 = \frac{(EA)(B)}{(EAB)}$$

Then, solving for (E),

$$(E) = \frac{Km1(EA)}{(A)} = \frac{Km2(EB)}{(B)}$$

Also, $$(EA) = \frac{Km2(EAB)}{(B)}$$

and $$(EB) = \frac{Km1(EAB)}{(A)}$$

Therefore, $$(E) = \frac{Km1Km2(EAB)}{(A)(B)}$$

Let the total enzyme concentration be E_t, that is,

$$(E)_t = (EAB) + (EB) + (EA) + (E)$$

then, $$(E)_t = (EAB) + \frac{Km1(EAB)}{(A)} + \frac{Km2(EAB)}{(B)} + \frac{Km1Km2(EAB)}{(A)(B)} \quad (1)$$

The final step in the reaction sequence is the breakdown of the ternary compound.

$$EAB \xrightarrow{k} E + \text{products}$$

The measured velocity is $v = k(EAB)$

Multiplying equation (1) by k, one obtains,

$$k(E)_t = k(EAB) + \frac{kKm1(EAB)}{(A)} + \frac{kKm2(EAB)}{(B)} + \frac{kKm1Km2(EAB)}{(A)(B)} \text{.}$$

Solving for k(EAB),

$$k(E)_t = k(EAB)\left[1 + \frac{Km1}{(A)} + \frac{Km2}{(B)} + \frac{Km1Km2}{(A)(B)}\right]$$

$$k(E)_t(A)(B) = k(EAB)\left[(A)(B) + Km1(B) + Km2(A) + Km1Km2\right]$$

$$k(EAB) = \frac{k(E)t(A)(B)}{(A)(B) + Km1(B) + Km2(A) + Km1Km2}$$

Then, substituting v for k(EAB),

$$v = \frac{k(E)t(A)(B)}{(A)(B) + Km1(B) + Km2(A) + Km1Km2}$$

The maximal velocity, Vx, will be attained when all the enzyme is bound by the substrate, and

$$(EAB) = (E)t$$

Under these circumstances,

$$Vx = k(EAB) = k(E)t$$

Thus, $$v = \frac{Vx(A)(B)}{(A)(B) + Km1(B) + Km2(A) + Km1Km2}$$

Inverting,

$$\frac{1}{v} = \frac{(A)(B) + Km1(B) + Km2(A) + Km1Km2}{Vx(A)(B)}$$

which becomes,

$$\frac{1}{v} = \frac{1}{Vx} + \frac{Km1}{Vx(A)} + \frac{Km2}{Vx(B)} + \frac{Km1Km2}{Vx(A)(B)} \qquad (2)$$

If the concentration of one substrate, (A), is varied while the other, (B), is held constant, equation (2) may be written,

$$\frac{1}{v} = \left\{\frac{(B) + Km2}{Vx(B)}\right\} + \frac{1}{(A)}\left\{\frac{Km1\left[(B) + Km2\right]}{Vx(B)}\right\} \qquad (3)$$

This is the equation of a straight line. If the slope of the straight line is divided by the ordinate intercept, the value for Km1 is obtained.

A straight line is obtained if (B) is varied while (A) is held constant,

$$\frac{1}{v} = \left\{\frac{(A) + Km1}{Vx(A)}\right\} + \frac{1}{(B)}\left\{\frac{Km2\left[(A) + Km1\right]}{Vx(A)}\right\} \qquad (4)$$

and values of maximum velocity, Vm2, and for Km2 can be determined.

If the concentrations of both substrates are varied and kept equal, (A) = (B) = (S)

and $$\frac{1}{v} = \frac{1}{Vx} + \frac{Km2}{Vx(S)} + \frac{Km1}{Vx(S)} + \frac{Km1Km2}{Vx(S)^2} \qquad (5)$$

It is evident that, in this case, the relation between 1/v and 1/(S) is not linear.

When only one substrate is varied, a plot of its concentration against the reaction velocities results

in a rectangular hyperbola. However, when both substrates are held equal and varied, a similar plot results in a sigmoidal curve.

3. Results

Experimental: To determine the effect of substrate concentration on intestinal GOT and GPT, experiments were carried out using both the colorimetric and spectrophotometric assay procedures. The intestinal homogenates, which were used as the source of enzyme, were prepared as described previously. A pH of 8.7 was used for the reactions being determined spectrophtometrically while pH 8.5 was used for reactions which were assayed colorimetrically. The phosphate concentration, however, was maintained at 0.10 M in both methods and all reactions were carried out at 37°C. Each set of results is the average of two experiments.

Varying the concentrations of both substrates of the GOT reaction simultaneously (spectrophotometric procedure): The results of the experiment, in which the concentrations of L-aspartate and α-ketoglutarate were kept equal and varied over a concentration range of 0.36 to 40 micromoles per ml., are presented in Table XI. In Fig. 20 the initial reaction velocities are plotted against the substrate concentration and Fig. 21 is a double reciprocal plot of the same data.

in a rectangular hyperbola. However, when both substrates are held equal and varied, a [illegible] results in a sigmoidal curve.

3. Results

Experimental: To determine the effect of substrate concentration on intestinal [illegible] and [illegible], experiments were carried out using both the colorimetric and spectrophotometric assay procedures. The intestinal homogenates, which were used as the source of enzyme, were prepared as described previously. A pH of 8.7 was used for the reactions being determined spectrophotometrically while pH 9.3 was used for reactions which were assayed colorimetrically. The inorganic concentrations, however, was maintained at 2.10 M in each reaction and all reactions were carried out at 37°C. Each set of results is the average of two experiments.

Studies on concentration of both substrates of the [illegible] reaction simultaneously (unidirectional assay): The results of the experiment, in which the concentrations of L-aspartate and [illegible] were kept equal and varied over a concentration range of 0.25 to 4.0 micromoles per ml., are presented in Table 21. In Fig. 20 the initial reaction velocities are plotted against the substrate concentration and Fig. 21 is a double reciprocal plot of the same data.

TABLE XI

The Effect of Varying the Concentrations of Both Substrates Simultaneously on the Initial Velocity of the GOT Reaction. Reaction System: temperature, 37°C.; pH, 8.7; buffer concentration, 0.10 M; enzyme concentration, 0.5 ml. homogenate; total volume, 3.0 ml.

Substrate Concentration, µM per ml.	Initial Velocity (X 10^{-2}), µM/ml./min.	$\frac{1}{(S)}$	$\frac{1}{v}$
0.36	0.25	2.78	4.00
0.40	0.30	2.50	3.33
0.50	0.41	2.00	2.44
0.60	0.49	1.67	2.05
0.80	0.59	1.25	1.70
1.20	0.84	0.83	1.19
1.60	0.97	0.63	1.03
2.40	1.22	0.42	0.82
4.00	1.51	0.25	0.66
6.00	1.92	0.17	0.52
12.00	2.45	0.08	0.41
16.00	2.62	0.06	0.38
24.00	2.90	0.04	0.34
32.00	3.10	0.03	0.32
40.00	3.29	0.03	0.30

TABLE [illegible]

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

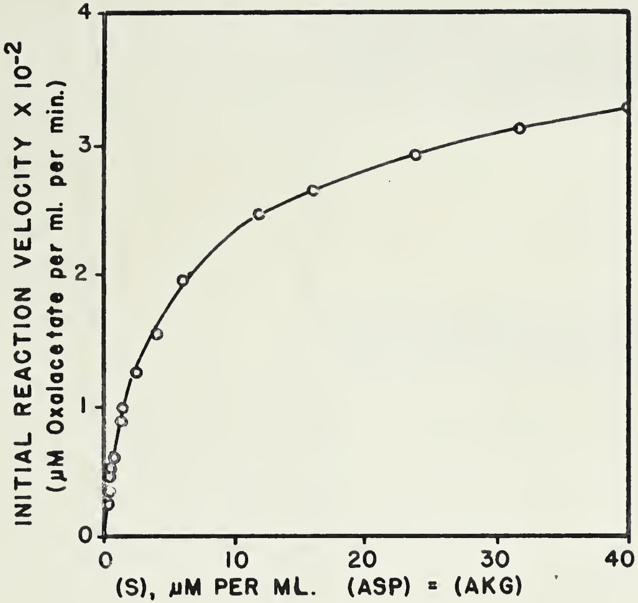

Fig. 20. The effect of varying the concentrations of both substrates simultaneously on the initial velocity of GOT.

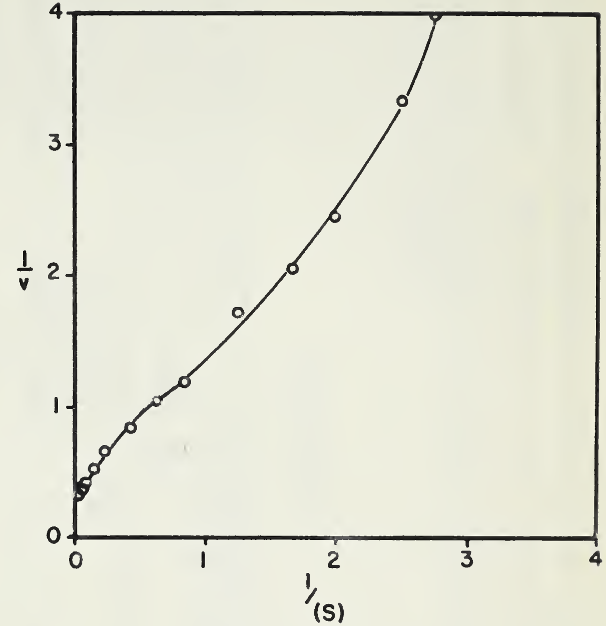

Fig. 21. Relation between reciprocals of initial velocity and substrate concentration for GOT reaction. Substrates varied simultaneously (ASP) = (AKG)

<u>Effect of varying only the concentration of α-ketoglutarate on the initial velocity of GOT (spectrophotometric procedure)</u>: The results of the experiment are presented in Table XII and direct and double reciprocal plots are shown in Figs. 22 and 23.

TABLE XII

The Effect of Varying the Concentration of α-Ketoglutarate on the Initial Velocity of the GOT Reaction. The Concentration of L-Aspartate is Held Constant at 40 μM per ml. Reaction System as in Table XI.

Substrate Concentration, μM per ml.	Initial Velocity (X 10^{-2}), μM/ml./min.	$\frac{1}{(S)}$	$\frac{1}{v}$
0.36	0.56	2.78	1.78
0.40	0.60	2.50	1.67
0.50	0.76	2.00	1.32
0.60	0.81	1.67	1.24
0.80	0.97	1.25	1.03
1.20	1.15	0.83	0.87
1.60	1.32	0.63	0.76
2.40	1.57	0.42	0.64
4.00	1.90	0.25	0.53
6.00	2.20	0.17	0.45
12.00	2.78	0.08	0.36
16.00	2.94	0.06	0.34
24.00	3.15	0.04	0.32
32.00	3.26	0.03	0.31
40.00	3.28	0.03	0.31

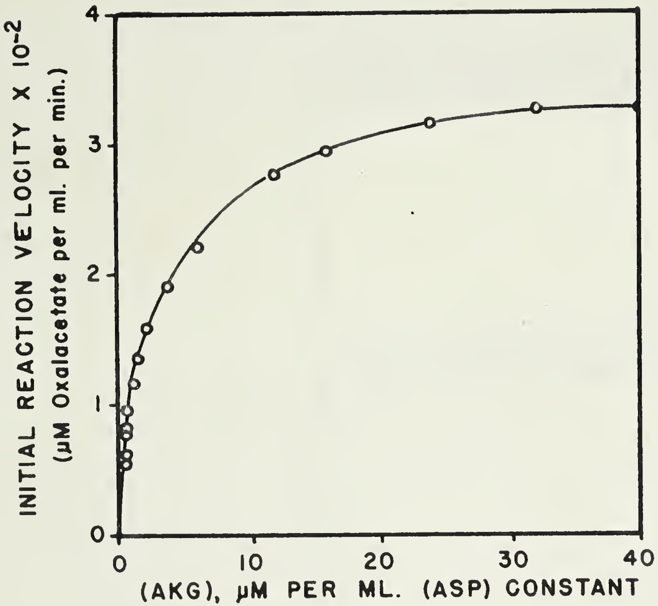

Fig. 22. The effect of varying the concentration of α-ketoglutarate on the initial velocity of GOT. Concentration of L-aspartate held constant at 40 µM per ml. reaction mixture.

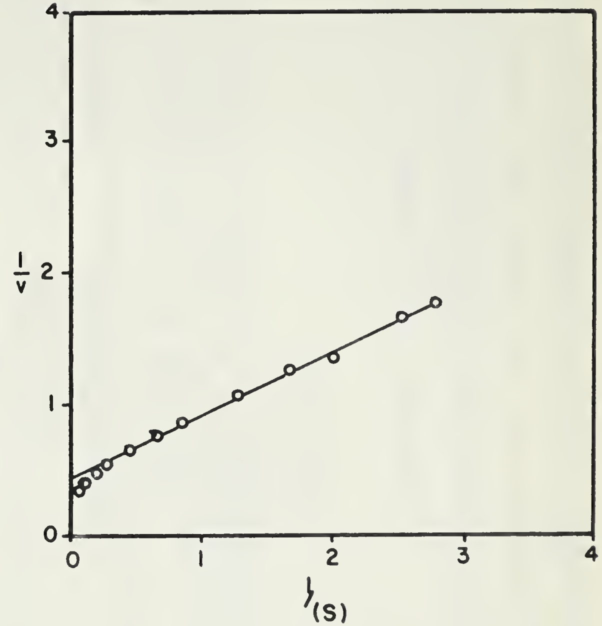

Fig. 23. Relation between reciprocals of initial velocity and substrate concentration for GOT reaction. (AKG) varied, (ASP) held constant at 40 µM per ml. reaction mixture.

<u>Effect of varying only the concentration of aspartate on the initial velocity of GOT (spectrophotometric procedure)</u>: The results of the experiment are presented in Table XIII while direct and double reciprocal plots of the data are shown in Figs. 24 and 25.

TABLE XIII

The Effect of Varying the Concentration of Aspartate on the Initial Velocity of the GOT Reaction. The Concentration of α-Ketoglutarate is Held Constant at 40 μM per ml. Reaction System as in Table XI.

Substrate Concentration, μM per ml.	Initial Velocity ($X\ 10^{-2}$), μM/ml./min.	$\frac{1}{(S)}$	$\frac{1}{v}$
0.36	0.86	2.78	1.16
0.40	0.97	2.50	1.03
0.50	1.14	2.00	0.88
0.60	1.27	1.67	0.79
0.80	1.41	1.25	0.71
1.20	1.66	0.83	0.60
1.60	1.84	0.63	0.54
2.40	2.06	0.42	0.49
4.00	2.32	0.25	0.43
6.00	2.51	0.17	0.40
12.00	2.91	0.08	0.34
16.00	3.10	0.06	0.32
24.00	3.20	0.04	0.31
32.00	3.30	0.03	0.30
40.00	3.30	0.03	0.30

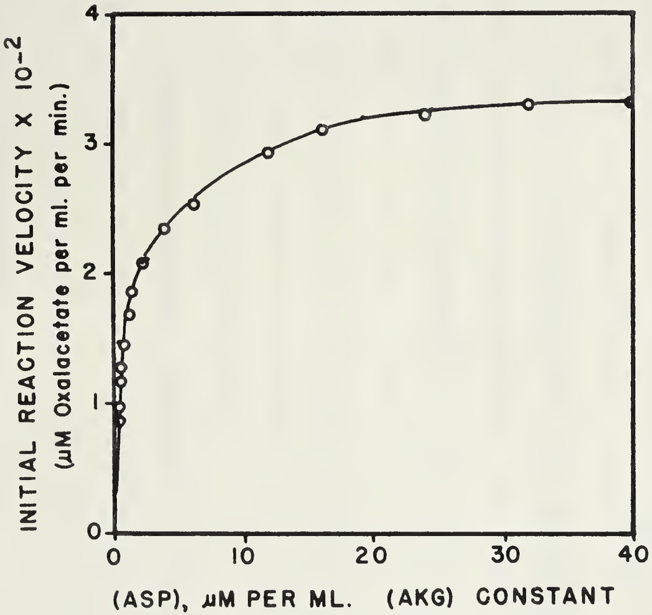

Fig. 24. The effect of varying the concentration of L-aspartate on the initial velocity of GOT. Concentration of α-ketoglutarate held constant at 40 μM per ml. reaction mixture.

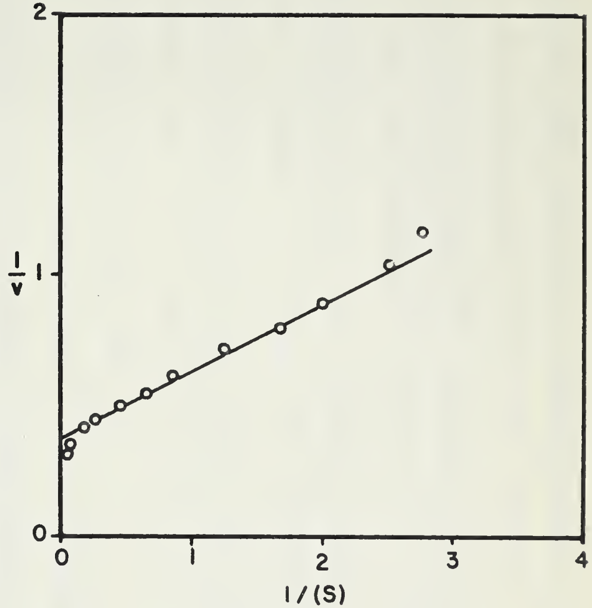

Fig. 25. Relation between reciprocals of initial velocity and substrate concentration. (ASP) varied, (AKG) held constant at 40 μM per ml. reaction mixture.

Varying the concentrations of both substrates of the GOT and GPT reactions simultaneously (colorimetric procedure): In determining the effect of substrate concentration on intestinal GOT and GPT colorimetrically, the concentrations of the substrates were varied over a range of 0.4 to 100 micromoles per ml. of reaction mixture. The results which were obtained by varying both substrates, while their concentrations were kept equal, are presented in Table XIV. Direct and double reciprocal plots of the data are shown in Figs. 26, 27, 28, and 29.

Effect of varying only the concentration of α-ketoglutarate on the activity of intestinal GOT and GPT (colorimetric procedure): The results of the experiments are presented in Table XV while direct and double reciprocal plots are shown in Figs. 30, 31, 32, and 33.

Effect of varying only the concentration of L-aspartate and L-alanine on the activity of intestinal GOT and GPT (colorimetric procedure): The results are presented in Table XVI. In Figs. 34 and 35 the enzyme activities are plotted against substrate concentration while in Figs. 36 and 37 the enzyme activities and substrate concentrations are plotted as reciprocals.

Michaelis-Menten constants: The Km constants which were obtained from the above data are presented in Table XVII. The method Dixon (40) was employed for determination of the Km constants.

TABLE XIV

The Effect of Varying the Concentrations of Both Substrates Simultaneously on the Activity of Intestinal GOT and GPT. Reaction System: temperature, 37°C.; pH, 8.5; buffer concentration, 0.10 M; enzyme concentration, 0.2 ml. intestinal homogenate; total volume, 1.5 ml.; incubation time, 30 minutes.

Substrate Concentration, µM per ml. Reaction Mixture.	GOT Activity, µM Oxal-acetate per ml. per 30 min.	GPT Activity, µM Pyruvate per ml. per 30 min.	$\frac{1}{(S)}$	GOT $\frac{1}{v}$	GPT $\frac{1}{v}$
0.4	0.05	0.06	2.50	18.8	16.7
0.6	0.08	0.09	1.67	13.2	11.1
0.8	0.09	0.11	1.25	11.1	9.1
1.0	0.12	0.13	1.00	8.3	7.7
1.2	0.15	0.15	0.83	6.7	6.7
2.0	0.20	0.22	0.50	5.0	4.5
4.0	0.31	0.36	0.25	3.3	2.8
8.0	0.40	0.54	0.13	2.5	1.9
12.0	0.47	0.66	0.08	2.4	1.5
20.0	0.54	0.79	0.05	1.8	1.3
40.0	0.64	0.90	0.03	1.6	1.1
60.0	0.65	0.90	0.02	1.6	1.1
100.0	0.65	0.90	0.01	1.6	1.1

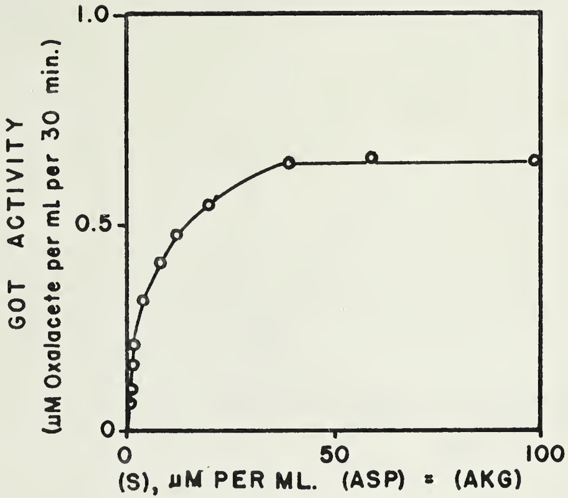

Fig. 26. The effect of varying the concentrations of both substrates simultaneously on the activity of intestinal GOT.

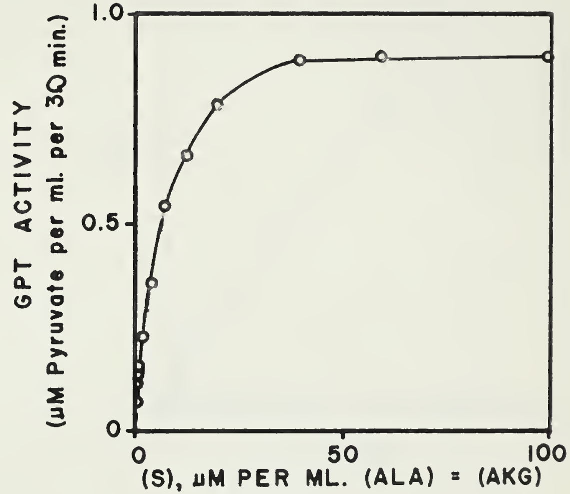

Fig. 27. The effect of varying the concentrations of both substrates simultaneously on the activity of intestinal GPT.

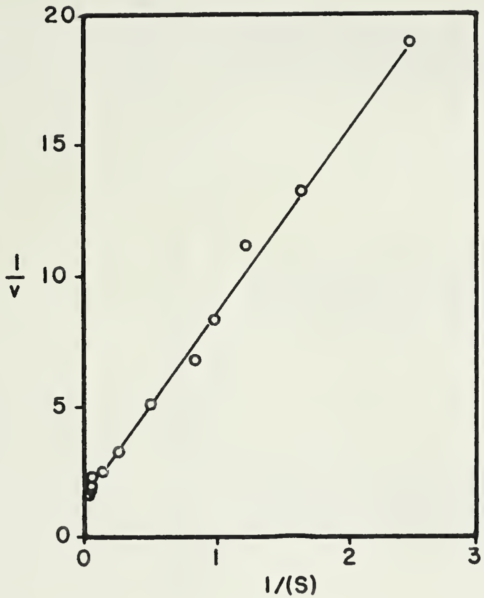

Fig. 28. Relation between reciprocals of activity and substrate concentration for GOT reaction. Substrates varied simultaneously. (ASP) = (AKG)

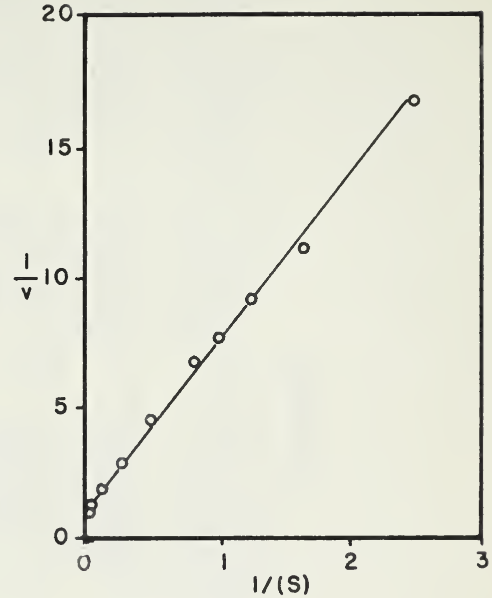

Fig. 29. Relation between reciprocals of activity and substrate concentration for GPT reaction. Substrates varied simultaneously. (ALA) = (AKG)

TABLE XV

The Effect of Varying the Concentration of α-Ketoglutarate and Keeping the Concentrations of L-Aspartate and L-Alanine Constant on the Activity of Intestinal GOT and GPT. Reaction System as in Table XIV.

Substrate Concentration, µM per ml. Reaction Mixture.	GOT Activity, µM Oxalacetate per ml. per 30 min.	GPT Activity, µM Pyruvate per ml. per 30 min.	$\frac{1}{(S)}$	GOT $\frac{1}{v}$	GPT $\frac{1}{v}$
0.4	0.13	0.30	2.50	7.8	3.3
0.6	0.18	0.42	1.67	5.7	2.4
0.8	0.21	0.47	1.25	4.7	2.1
1.0	0.25	0.54	1.00	4.0	1.9
1.2	0.27	0.58	0.83	3.7	1.7
2.0	0.33	0.71	0.50	3.0	1.4
4.0	0.41	0.80	0.25	2.4	1.3
8.0	0.50	0.87	0.13	2.0	1.2
12.0	0.56	0.89	0.08	1.8	1.1
20.0	0.60	0.90	0.05	1.7	1.1
40.0	0.60	0.90	0.03	1.7	1.1
60.0	0.61	0.90	0.02	1.6	1.1
100.0	0.61	0.90	0.01	1.6	1.1

Concentration of L-Aspartate and L-Alanine Held at 60 µM per ml.

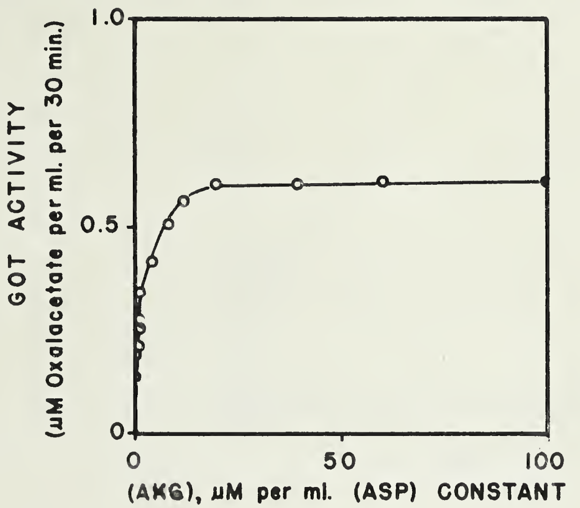

Fig. 30. The effect of varying the concentration of α-ketoglutarate on the activity of GOT. Concentration of L-aspartate held constant at 60 µM per ml. reaction mixture.

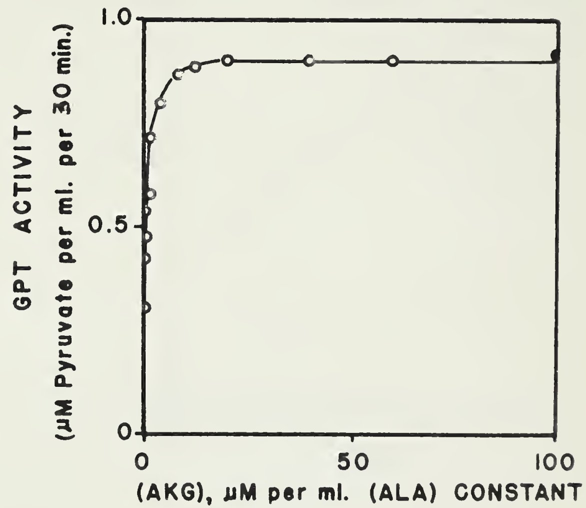

Fig. 31. The effect of varying the concentration of α-ketoglutarate on the activity of GPT. Concentration of L-alanine held constant at 60 µM per ml. reaction mixture.

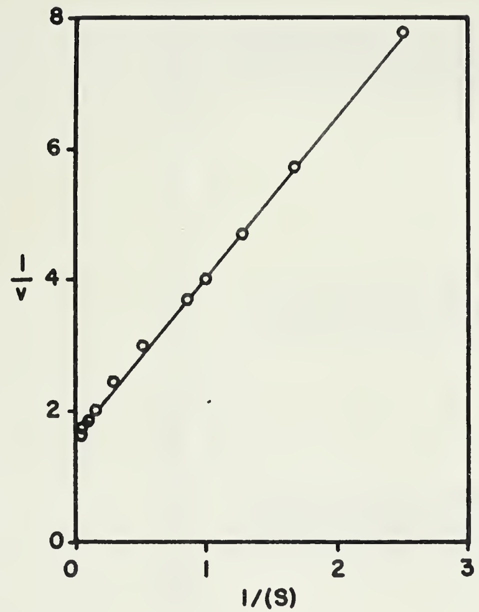

Fig. 32. Relation between reciprocals of activity and substrate concentration for GOT reaction. (AKG) varied, (ASP) held constant at 60 μM per ml. reaction mixture.

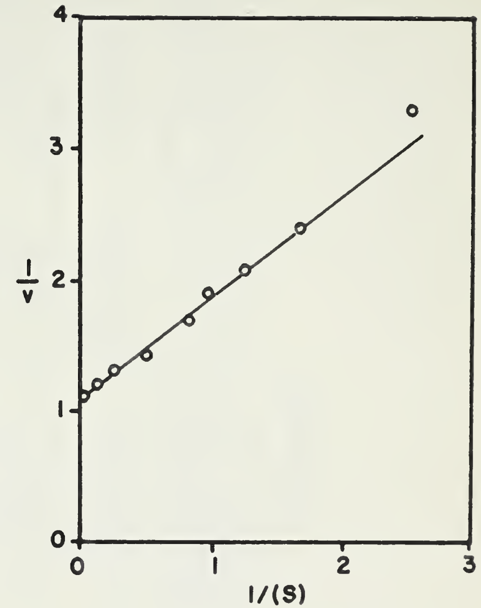

Fig. 33. Relation between reciprocals of activity and substrate concentration of GPT reaction. (AKG) varied, (ALA) held constant at 60 μM per ml. reaction mixture.

TABLE XVI

The Effect of Varying the Concentration of L-Aspartate and L-Alanine and Keeping the Concentration of α-Keto-glutarate Constant on the Activity of Intestinal GOT and GPT. Reaction System as in Table XIV.

Substrate Concentration, µM per ml. Reaction Mixture.	GOT Activity, µM Oxalacetate per ml. per 30 min.	GPT Activity, µM Pyruvate per ml. per 30 min.	$\frac{1}{(S)}$	GOT $\frac{1}{v}$	GPT $\frac{1}{v}$
0.4	0.13	0.15	2.50	7.8	6.6
0.6	0.17	0.22	1.67	5.9	4.5
0.8	0.20	0.26	1.25	5.0	3.9
1.0	0.24	0.29	1.00	4.2	3.5
1.2	0.27	0.32	0.83	3.7	3.1
2.0	0.34	0.41	0.50	2.9	2.4
4.0	0.42	0.54	0.25	2.4	1.9
8.0	0.49	0.69	0.13	2.0	1.5
12.0	0.53	0.76	0.08	1.9	1.3
20.0	0.59	0.83	0.05	1.7	1.2
40.0	0.63	0.90	0.03	1.6	1.1
60.0	0.63	0.91	0.02	1.6	1.0
100.0	0.63	0.90	0.01	1.6	1.1

Concentration of α-Keto glutarate Held at 20 µM per ml.

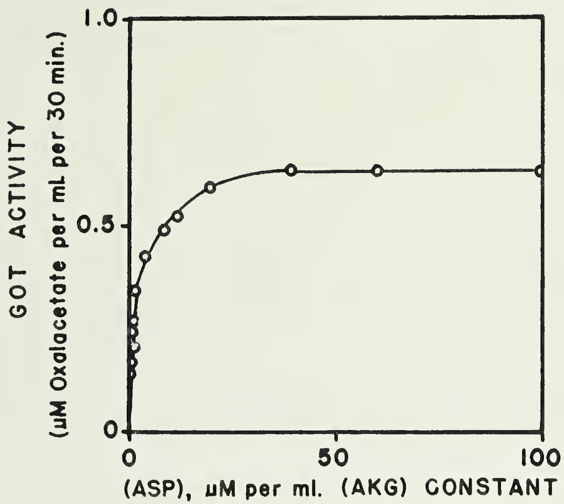

Fig. 34. The effect of varying the concentration of L-aspartate on the activity of GOT. Concentration of α-ketoglutarate held constant at 20 µM per ml. reaction mixture.

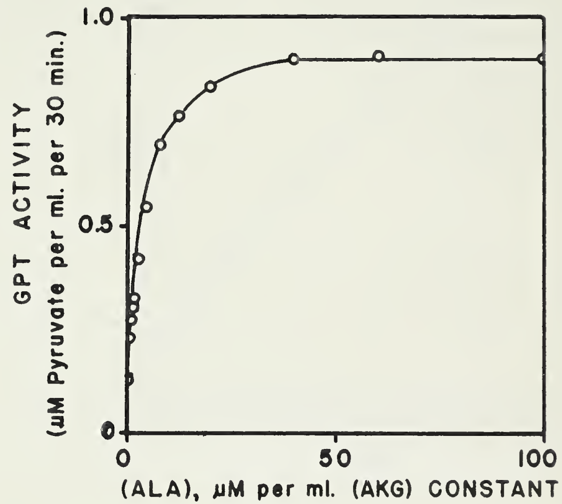

Fig. 35. The effect of varying the concentration of L-alanine on the activity of GPT. Concentration of α-ketoglutarate held constant at 20 µM per ml. reaction mixture.

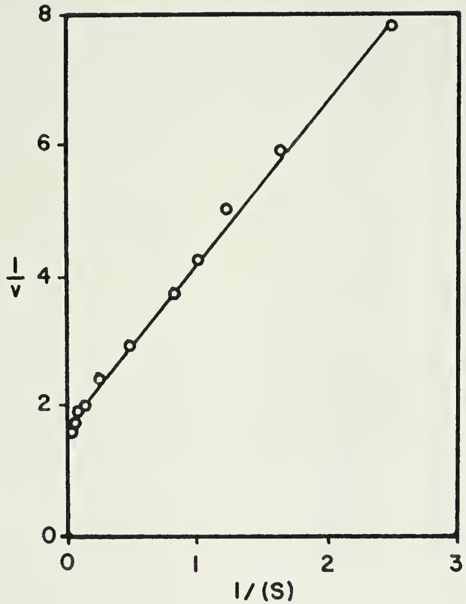

Fig. 36. Relation between reciprocals of activity and substrate concentration for GOT reaction. (ASP) varied, (AKG) held constant at 20 µM per ml. reaction mixture.

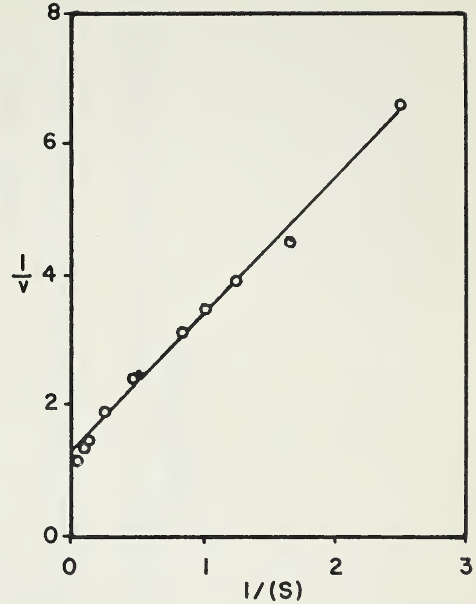

Fig. 37. Relation between reciprocals of activity and substrate concentration for GPT reaction. (ALA) varied, (AKG) held constant at 20 µM per ml. reaction mixture.

TABLE XVII

Michaelis-Menten Constants of Intestinal GOT and GPT.

Experiment	Km_1 µM/ml.	Km_2 µM/ml.
Spectrophotometric procedure (GOT)		
(ASP) varied, (AKG) held constant	0.70	
(AKG) varied, (ASP) held constant		1.20
Colorimetric procedure (GOT)		
(ASP) varied, (AKG) held constant	1.52	
(AKG) varied, (ASP) held constant		1.50
Colorimetric procedure (GPT)		
(ALA) varied, (AKG) held constant	1.72	
(AKG) varied, (ALA) held constant		0.72

4. Discussion

Equation 5 of the mathematical treatment showed that when the concentrations of both substrates are held equal and varied, a sigmoidal curve results. Such a curve was, in fact, obtained (Fig. 20) when reaction velocities were measured spectrophotometrically. When the enzyme activity was measured colorimetrically, the same experiment produced parabolic curves (Figs. 26, 27). Two possible reasons for this discrepancy may be that the colorimetric procedure does not measure initial reaction rates under non-optimal conditions, and that, as shown earlier while determining the effect of pH, some denaturation of the enzyme occurs during the 30 minute incubation period. In all experiments in which only the concentration of one substrate was varied, the expected parabolic curves were obtained when the velocity was plotted against the substrate concentration. Also, when the reciprocals of the velocity and substrate concentration were plotted, straight-line relationships were found.

In his studies with corn radicle GOT, Cook (35) found Km1 to be 0.93 µM/ml. and Km2 to be 1.33 µM/ml. Similar results (Km1 = 0.70 µM/ml.; Km2 = 1.20 µM/ml.) were obtained in our study. This indicates that intestinal GOT combines somewhat more readily with aspartic acid than with α-keto-glutarate. Use of the colorimetric procedure, however,

resulted in values of Km1 = 1.52 μM/ml. and Km2 = 1.50 μM/ml. for GOT and Km1 = 1.72 μM/ml. and Km2 = 0.72 μM/ml. for GPT. Thus it could be concluded that GOT combines with both substrates with approximately equal affinity while GPT combines with greater affinity with α-ketoglutarate than with alanine. In view of the probability, however, that the colorimetric procedure does not measure initial reaction velocities at lower substrate concentrations, little meaning should be assigned to these Km values. The experiments in which the relation between enzyme activity and substrate concentration were determined colorimetrically did provide valuable information, however, for the results obtained confirm the preliminary studies that a substrate concentration of 60 μM/ml. reaction mixture of amino acid and 20 μM/ml. reaction mixture of α-ketoglutaric acid should be used for assaying GOT and GPT routinely.

D. Effect of Enzyme Concentration

To study the effect of enzyme concentration, intestinal homogenate was prepared as described previously and then GOT and GPT assays were carried out on reaction mixtures containing 0.0 to 0.6 ml. homogenate. The reaction mixtures contained 90 micromoles amino acid and 30 micromoles α-keto-glutarate per 1.5 ml. and were buffered to pH 8.5 with 0.10 M phosphate buffer. The salicylaldehyde method was employed for assay purposes.

The results, which are an average of two experiments, are presented in Table XVIII. Fig. 38 demonstrates that the relationship between enzyme activity and enzyme concentration is linear for GOT for concentrations of homogenate from 0.0 to 0.4 ml. per 1.5 ml. reaction mixture. For GPT, on the other hand, the relationship is linear for homogenate concentrations from 0.0 to 0.6 ml. per 1.5 ml. reaction mixture.

It was considered satisfactory, therefore, to use 0.2 ml. homogenate per 1.5 ml. reaction mixture for routine transaminase assays.

TABLE XVIII

The Effect of Enzyme Concentration on the Activity of Intestinal GOT and GPT of the Rat. Reaction System: 90 µM amino acid, 30 µM α-ketoglutarate buffered to pH 8.5 with 0.10 M phosphate buffer. Temperature, 37°C. Incubation time, 30 minutes.

Ml. Homogenate per 1.5 ml. Reaction Mixture	GOT Activity, µM per ml. per 30 min.	GPT Activity, µM per ml. per 30 min.
0.025	0.05	0.06
0.050	0.10	0.13
0.075	0.15	0.19
0.100	0.20	0.26
0.150	0.30	0.40
0.200	0.40	0.52
0.250	0.50	0.66
0.300	0.58	0.78
0.400	0.78	1.04
0.500	0.93	1.32
0.600	1.06	1.56

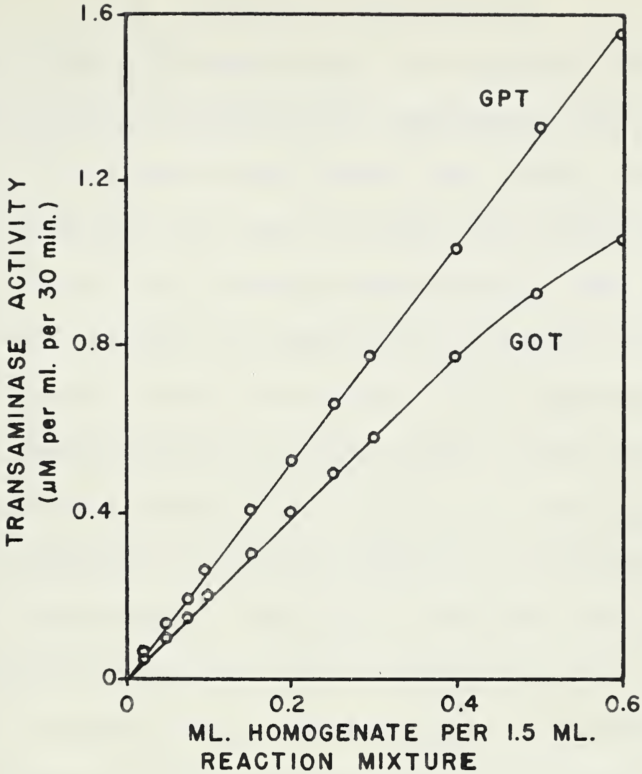

Fig. 38. The effect of enzyme concentration on the activity of intestinal GOT and GPT of the rat.

E. Effect of Temperature

The effect of temperature on intestinal GOT and GPT was determined by varying the temperature over the range 5 - 40°C. At temperatures lower than 20°C., longer incubation periods were used in order that sufficient pyruvate would be formed for accurate measurement. The reaction systems were exactly as described in chapter II (description of colorimetric assay procedure). The experiments were repeated twice and the average values of the two experiments are presented in Table XIX. In Fig. 39 the logarithm of the reaction velocity is plotted against the reciprocal of the absolute temperature. Between 5 and 40°C. the data are well represented by straight lines, indicating that the arrhenius equation is applicable. The energies of activation, calculated from the slope of the lines, were found to be 8,800 calories per mole for GOT and 9,170 calories per mole for GPT.

Nisonoff and Barnes (107) found that the energy of activation of partially purified GOT of hog heart is 12,500 calories per mole. Their data were represented by a straight line between 13 and 40°C. Darling (37), who worked with a partially purified GPT of ox heart, found that, when the logarithm of the reaction velocity was plotted against the reciprocal of the absolute temperature, a sharp break in the relationship occured. He found the reaction to follow

[illegible]

[illegible]

[illegible]

the Arrhenius equation accurately from 0 - 40°C. with an energy of activation of 17,475 calories per mole and from 34 - 70°C with an energy of activation of 8,740 calories per mole. Thus, GPT of ox hearts appears to possess a critical temperature at 34°C. On examination of the data of Nisonoff and Barnes (107), a similar break in the straight line relationship was observed to occur at 40°C.

Had the transaminase assays in our studies been carried out at temperatures exceeding 40°C., intestinal GOT and GPT also may have been shown to possess critical temperatures. Sizer (134) has suggested that such a change in energy of activation represents a shift in the configuration of the enzyme molecule.

The large discrepancies between the energies of activation obtained in our laboratory and those obtained by Nisonoff and Barnes (107) and Darling (37) are probably due to such factors as purity of the enzyme preparation, source of enzyme, and difference of assay conditions.

TABLE XIX

The Effect of Temperature on the Reaction Rate of Intestinal GOT and GPT of the Rat.

Temperature, °C.	$1/T \times 10^{-4}$	GOT Log Reaction Rate	GPT Log Reaction Rate
5	36.0	-1.03	-0.95
10	35.3	-0.84	-0.81
15	34.7	-0.73	-0.68
20	34.1	-0.60	-0.56
25	33.5	-0.50	-0.44
30	33.0	-0.39	-0.33
34	32.6	-0.31	-0.26
37	32.3	-0.26	-0.20
40	32.0	-0.20	-0.15

[illegible]

F. Effect of Time

The effect of incubating GOT and GPT reaction mixtures for various time intervals (0 - 60 min.) was determined and the average results of two experiments are presented in Table XX. Fig. 40 clearly demonstrates that a linear relation exists between reaction rate and time of incubation in the range 0 - 60 minutes.

TABLE XX

The Effect of Time on the Reaction Rate of Intestinal GOT and GPT of the Rat.

Incubation Time (min.)	GOT Activity, µM per ml.	GPT Activity, µM per ml.
5	0.09	0.07
10	0.17	0.17
15	0.25	0.29
20	0.32	0.39
25	0.42	0.47
30	0.51	0.55
35	0.59	0.65
40	0.68	0.74
45	0.77	0.84
50	0.85	0.93
55	0.94	1.03
60	1.02	1.12

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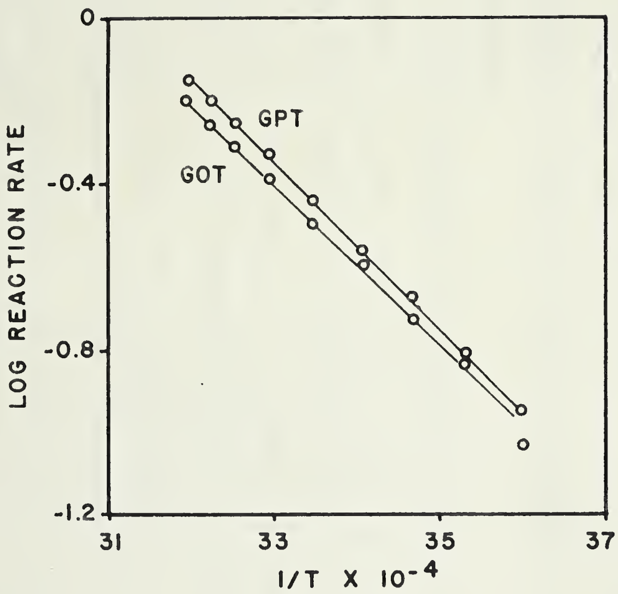


Fig. 39. The effect of temperature on the reaction rate of intestinal GOT and GPT of the rat.

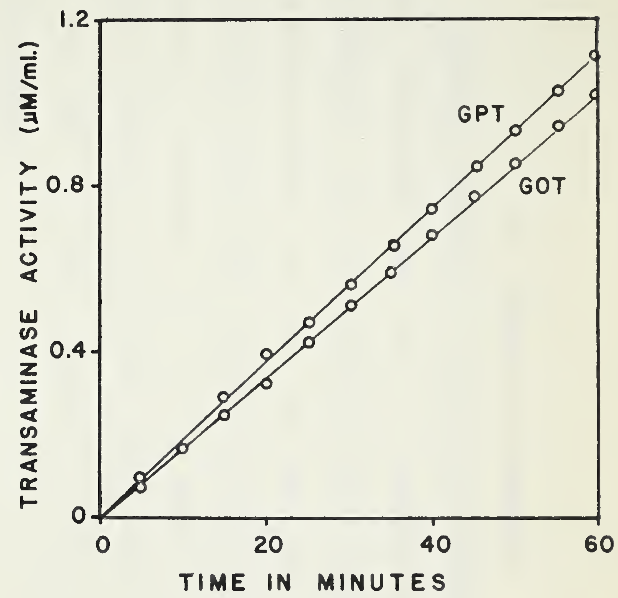


Fig. 40. The effect of time on the reaction rate of intestinal GOT and GPT of the rat.

G. Effect of Storage

Whenever storage of intestinal homogenates was necessary, they were kept in the freezing compartment of the refrigerator. As the occasion never arose to use an enzyme preparation which was more than four days old, the effect of storage beyond this length of time was not determined. No loss of activity was found to occur during a four day period. Nisonoff and Barnes (107) found that storage of pig heart GOT at -20°C. results in 2.5% loss of activity in six months.

H. Summary

Reaction kinetics of intestinal GOT and GPT have been determined by means of spectrophotometric and colorimetric procedures.

In the presence of phosphate buffer, maximum activity was shown to occur at pH 8.5 - 8.8, while in the presence of barbital buffer an extremely sharp pH optimum of 9.08 was found. Saturation of enzyme with substrate was shown to occur when the reaction mixture contains 90 micromoles amino acid, 30 micromoles α-ketoglutaric acid, and 0.2 ml. homogenate per 1.5 ml. The reaction rate was found to increase continuously with increasing temperature between 5 and 40°C.; the activation energies of GOT and GPT were

8,800 and 9,170 calories per mole respectively. Storage of intestinal homogenates for four days resulted in no loss of transaminase activity.

CHAPTER V

EFFECT OF FASTING

A. Introduction

Fasted animals were to be used for the study of the effect of force-feeding of various amino acids on transamination in the wall of the small intestine. It was necessary, therefore, to determine the changes which occur in the levels of intestinal transaminases and amino acids of starved rats.

Apparently there have been no reports in the literature dealing with the effect of fasting on transaminase activity in the small intestine of the rat. Triantas (141) found that the concentration of total free amino acids in the first 10 cm. (from the pylorus on) of the small intestine falls to 79% of the normal value in four days. This fall of amino acid concentration was shown to parallel roughly the weight loss of the intestine.

B. Experimental

All animals used in this experiment were housed in individual cages. The non-fasting animals were maintained on Purina fox checkers and water *ad libitum*, while fasting animals were allowed only water. The 34 rats which were used in the experiment were apportioned as follows:

non-fasting---------------- seven rats

one day of fasting --------- six rats

two days of fasting ---------- ten rats

three days of fasting -------- six rats

four days of fasting --------- five rats

At the end of the fasting period the rats were killed by decapitation and the second and third 10 cm. sections of the small intestine immediately were excised and cleaned as described previously. The second 10 cm. section was used for the determination of free amino acids and the third 10 cm. section was used for the assay of GOT and GPT activity. Amino acids were estimated by paper chromatography and transaminase assays were carried out by means of the salicylaldehyde procedure (analytical procedures described in chapter II).

C. Results and Discussion

The mean weights of the rats and of the second and third 10 cm. sections of intestine are tabulated in Table XXI. The data, which are presented on a per cent basis in Fig. 41, show that loss of body weight is not as rapid as the loss of weight of the intestine. A similar finding was reported by Triantas (141).

The transaminase activities and free amino acid levels which were found in rat intestine at various stages of fasting are presented in Table XXII.

Fig. 42 is a graphical representation of the effect

TABLE XXI

The Effect of Fasting on Body Weight and Weight of Intestinal Sections

Period of Fasting (Days)	Initial Body Weight (gm.)	Weight Loss (gm.)	Weight of 2nd 10 cm. Section (gm.)	Weight of 3rd 10 cm. Section (gm.)
0	280 ± 3*	0	0.59 ± 0.03	0.60 ± 0.03
1	270 ± 3	18 ± 1	0.50 ± 0.03	0.53 ± 0.02
2	280 ± 5	37 ± 1	0.44 ± 0.01	0.47 ± 0.02
3	289 ± 5	47 ± 2	0.42 ± 0.003	0.41 ± 0.01
4	262 ± 4	52 ± 2	0.34 ± 0.01	0.40 ± 0.01

* Standard error of the mean.

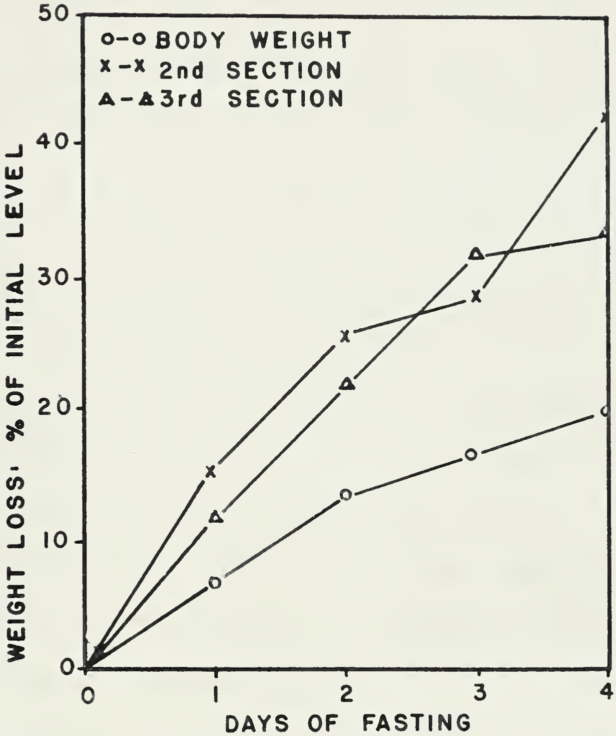


Fig. 41. The effect of fasting on body weight and weight of 2nd and 3rd intestinal sections.

of fasting on intestinal transaminase activity. When intestinal weights of fasted rats are used for calculating the units of transaminase activity, starvation appears to cause an increase of intestinal GOT and GPT activity which reaches a maximum after three days of fasting. The reason for this seeming anomaly is that during fasting the loss of weight of the intestine is proportionately more rapid than the loss of transaminase activity. Hence, the net effect shows an increase of transaminase activity. However, if a correction is made for weight losses of the intestine during fasting, transaminase levels are found to decrease rapidly during the first two days of fasting and then more gradually during the third and fourth days of food deprivation. Triantas (141) showed that intestinal alkaline phosphatase drops to 67% of the normal level after two days of fasting and then remains at a constant value until the fifth day. Intestinal GOT, as shown in the present study, falls to 77% of the normal value after two days of fasting and to 68% of normal after four days of fasting. Intestinal GPT decreases to 83% of the normal level after two days of fasting and to 79% of normal after four days of fasting.

In Figs. 43 and 44 the levels of free amino acids which were found in intestinal homogenates are plotted against the period of fasting. Although the patterns of the four amino acids are different, the general trend seems

TABLE XXII

The Effect of Fasting on GOT and GPT Activity and on Levels of Free Amino Acids in the Small Intestine of the Rat

Period of Fasting (Days)	Units per gm. Wet Intestine		Micrograms per gm. Wet Intestine			
	GOT	GPT	ASP	GLUT	GLYC	ALAN
0	718 ± 23*	894 ± 27	256 ± 25	545 ± 19	364 ± 8	403 ± 15
1	711 ± 29 (620 ± 13)	879 ± 5 (770 ± 25)	257 ± 27 (213 ± 27)	544 ± 33 (465 ± 42)	445 ± 16 (377 ± 17)	295 ± 38 (252 ± 35)
2	707 ± 13 (549 ± 13)	949 ± 20 (736 ± 17)	138 ± 15 (105 ± 12)	586 ± 13 (443 ± 14)	440 ± 34 (341 ± 21)	376 ± 31 (282 ± 17)
3	772 ± 10 (527 ± 5)	1159 ± 60 (771 ± 52)	141 ± 9 (100 ± 6)	513 ± 33 (365 ± 24)	422 ± 19 (299 ± 14)	276 ± 36 (195 ± 26)
4	724 ± 24 (487 ± 16)	1052 ± 41 (707 ± 32)	178 ± 15 (100 ± 9)	635 ± 32 (369 ± 17)	489 ± 17 (285 ± 11)	286 ± 22 (167 ± 13)

* Standard error of the mean

Brackets contain values which have been corrected for tissue weight losses due to fasting.

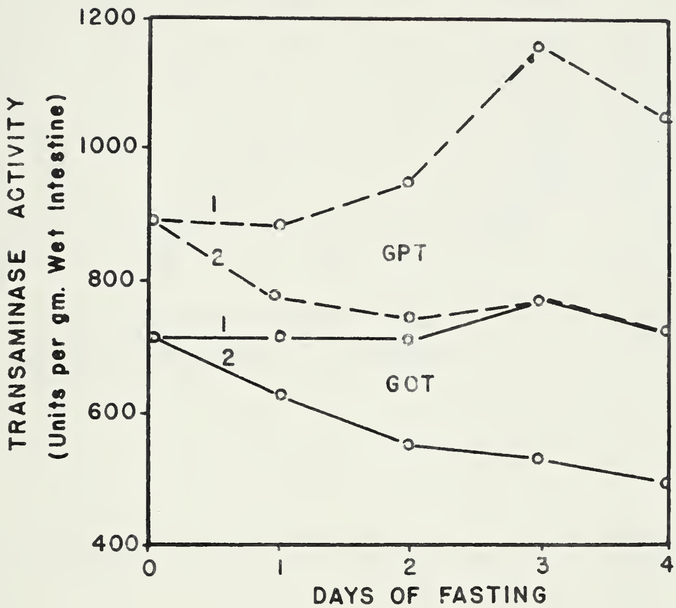


Fig. 42. The effect of fasting on intestinal GOT and GPT activity of the rat.

1. Uncorrected for weight losses of intestine.
2. Corrected for weight losses of intestine.

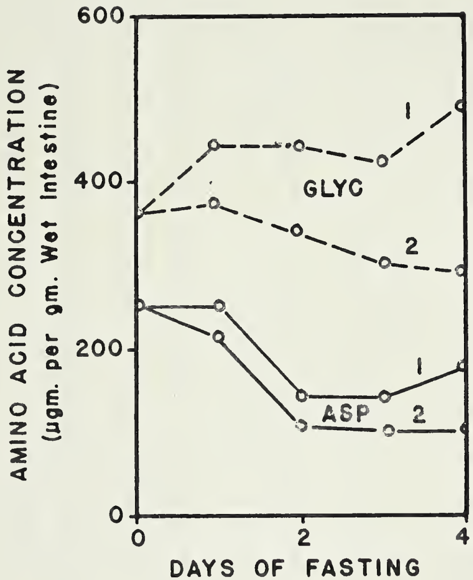


Fig. 43. The effect of fasting on levels of glycine and aspartic acid of the small intestine of the rat.

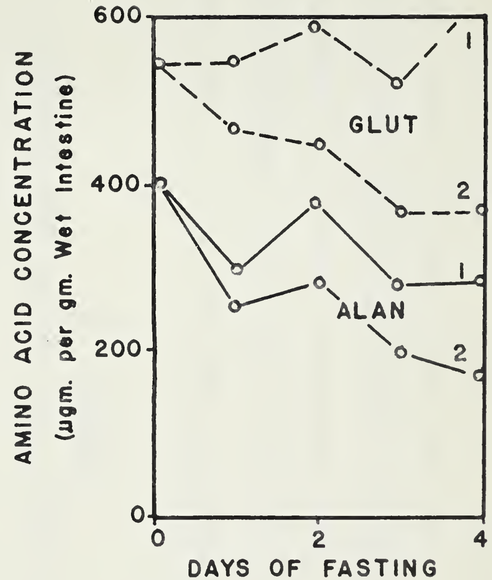


Fig. 44. The effect of fasting on levels of glutamic acid and alanine of the small intestine of the rat.

1. Uncorrected for weight losses of intestine.
2. Corrected for weight losses of intestine.

to be a gradual decrease of amino acid concentration throughout the four-day fasting period.

After three days of fasting, approximately 30% of the animals were observed to develop diarrheoa. Upon consideration of the above data and the observation that, after three days of fasting, food deprivation begins to cause unphysiological conditions, a two day fasting period was chosen for the force-feeding experiments.

D. Summary

1. Intestinal GOT activity was found to decrease to 77% of the normal value as a result of a two-day fast and to 68% of normal after an additional two days of fasting.
2. Intestinal GPT activity fell to 83% of the normal level after two days of fasting and to 79% of normal after four days of fasting.
3. The concentration of aspartic acid was found to fall to approximately half the normal value during a two-day fast while the concentrations of glutamic acid and glycine decreased more gradually to approximately three quarters of the normal values after four days of fasting. The concentration of alanine fell to less than 50% of normal by the end of four days of fasting.

CHAPTER VI

EFFECT OF AMINO ACID INGESTION ON TRANSAMINATION IN THE SMALL INTESTINE OF THE RAT

A. Introduction

Numerous workers have attempted to explain the mechanism by which amino acids are absorbed by the small intestine of mammals (149, 26, 10), but Hober and Hober (60) were the first to suggest that an active process may be involved. This suggestion was later confirmed by several investigators using both *in vivo* (132, 28, 89, 91, 78) and *in vitro* (51, 150, 1, 48, 151) techniques. Wiseman (150) showed that rat small intestine can transport a number of amino acids against a concentration gradient, while glutamic and aspartic acids are absorbed passively. Later, Wiseman and coworkers (90, 104, 105) provided evidence that glutamic and aspartic acids are involved in transamination reactions when absorbed by the small intestine of cats, rabbits, dogs, or rats.

After we had confirmed the reports of Wiseman and coworkers that transaminases are present in the small intestine of the rat, the following experiments were carried out in our laboratory in the hope of obtaining some clue as to the functions of intestinal GOT and GPT during the absorption of certain amino acids.

B. Experimental

All experiments were performed on adult male albino rats which weighed 240 - 300 grams. The animals were housed in individual cages and water was provided ad libitum during the four-day experiment. The rats were fasted for two days prior to the force-feeding of amino acid solutions.

The force-feeding technique of Triantas (141), which allows the rats to become accustomed to the gastric intubation, was found to be satisfactory for our experiments. Hence, it was adopted and carried out as follows.

At 9 a.m. and 5 p.m. of the third day of fasting, the rats were force-fed three ml. of 0.75 M solution of the amino acid under investigation. On the following day, the force-feedings were repeated at 8 a.m. and 1 a.m., and then at 1 p.m. the rats were killed by decapitation. The force-feeding apparatus consisted of a 5 ml. syringe which was attached to a catheter of 1.5 mm. diameter. All amino acid solutions were adjusted to pH 7. Although 0.75 M amino acid solutions are hypertonic, this concentration was chosen in order to cause more rapid changes, if any were to occur, in intestinal transaminase levels. The control animals were force-fed distilled water in place of amino acid solutions. Eleven animals constituted the control group and each amino acid solution was fed to nine rats.

Necropsy findings revealed that water, L-alanine solution, and glycine solution were absorbed almost completely, but the stomach of an animal which had been fed L-aspartate or L-glutamate contained approximately two ml. of fluid. This parallels the observation of Wiseman (150) that glycine and alanine are actively absorbed while aspartate and glutamate are passively absorbed. One additional observation of interest was made at the time of killing. Immediately after decapitation, normal rats exhibit violent spinal reflexes which consist of a running motion of the hind legs. These same reflexes were observed with rats which had been fed alanine and glycine. Aspartate- and glutamate-fed rats, on the other hand, displayed strikingly different reflex patterns. For approximately 15 seconds following decapitation, no movement whatsoever occurred. Then, gradually increasing in strength, a stretch reflex very similar to the kind displayed by strychnine-poisoned animals occurred. The possibility exists that glutamic and aspartic acids may block to a greater extent impulses from the spinal cord to the flexor muscles than to the extensor muscles. Further investigation is required, however, before an definite conclusions can be drawn.

Immediately after the animals had been killed, the second and third 10 cm. sections of the small intestine were removed and cleaned. The second section was used for estimation of free amino acids, and the third section was used for assaying GOT and GPT.

C. Results

The results of the experiment are presented in Table XXIII and in Fig. 45.

Force-feeding of 0.75 M glycine: Feeding of a 0.75 M glycine solution resulted in a depression of intestinal GOT and GPT activity to 85% of the control level. As expected, the concentration of intestinal glycine was much increased and although the concentrations of aspartate and alanine did not change significantly, the concentration of glutamic acid was found to fall to 79% of the control level.

Force-feeding of 0.75 M L-alanine: Significant losses of GOT and GPT activities were found to occur as a result of force-feeding 0.75 M L-alanine. The concentrations of L-glutamate and glycine remained unchanged while the concentrations of L-aspartate and L-alanine were increased.

Force-feeding of 0.75M L-glutamate: Although the ingestion of 0.75 M L-glutamate resulted in no change of transaminase levels, highly significant changes were found in the levels of free amino acids. The concentrations of L-aspartate, L-glutamate, and L-alanine all showed an increase. The concentration of glycine, however, showed a striking decrease.

TABLE XXIII

The Effect of Amino Acid Ingestion on the Activity of Intestinal GOT and GPT and on Levels of Intestinal Free Amino Acids of the Rat.

Amino Acid Fed	Initial Body Weight (gm.)	Weight Loss (gm.)	Transaminase Activity, Units per gm. Wet Tissue		Amino Acid Concentration, Micrograms per gm. Wet Tissue			
			GOT	GPT	ASP	GLUT	GLYC	ALAN
Controls (11)	285 ± 5*	46 ± 2	746 ± 13	1136 ± 41	170 ± 11	586 ± 35	463 ± 18	291 ± 22
Glycine (9)	274 ± 3	40 ± 2	631 ± 24 $p < 0.01$	966 ± 13 $p < 0.01$	181 ± 19 $p > 0.5$	462 ± 22 $p < 0.01$	1446 ± 90 $p < 0.01$	312 ± 31 $p > 0.5$
L-Alanine (9)	266 ± 6	40 ± 1	699 ± 11 $p < 0.02$	1011 ± 33 $p < 0.05$	235 ± 24 $p < 0.05$	527 ± 29 $p > 0.1$	457 ± 12 $p > 0.5$	522 ± 42 $p < 0.01$
L-Glutamic (9)	278 ± 7	46 ± 1	726 ± 11 $p > 0.1$	1119 ± 46 $p > 0.5$	253 ± 23 $p < 0.01$	2711 ± 168 $p < 0.01$	346 ± 15 $p < 0.01$	463 ± 36 $p < 0.01$
L-Aspartic (9)	264 ± 4	47 ± 1	719 ± 19 $p > 0.1$	1019 ± 37 $p < 0.05$	1019 ± 37 $p < 0.01$	740 ± 42 $p < 0.02$	371 ± 20 $p < 0.01$	780 ± 17 $p < 0.01$

* Standard error of the mean.
Enzyme values are not corrected for tissue weight loss due to fasting.
Numbers in brackets indicate the number of rats used.

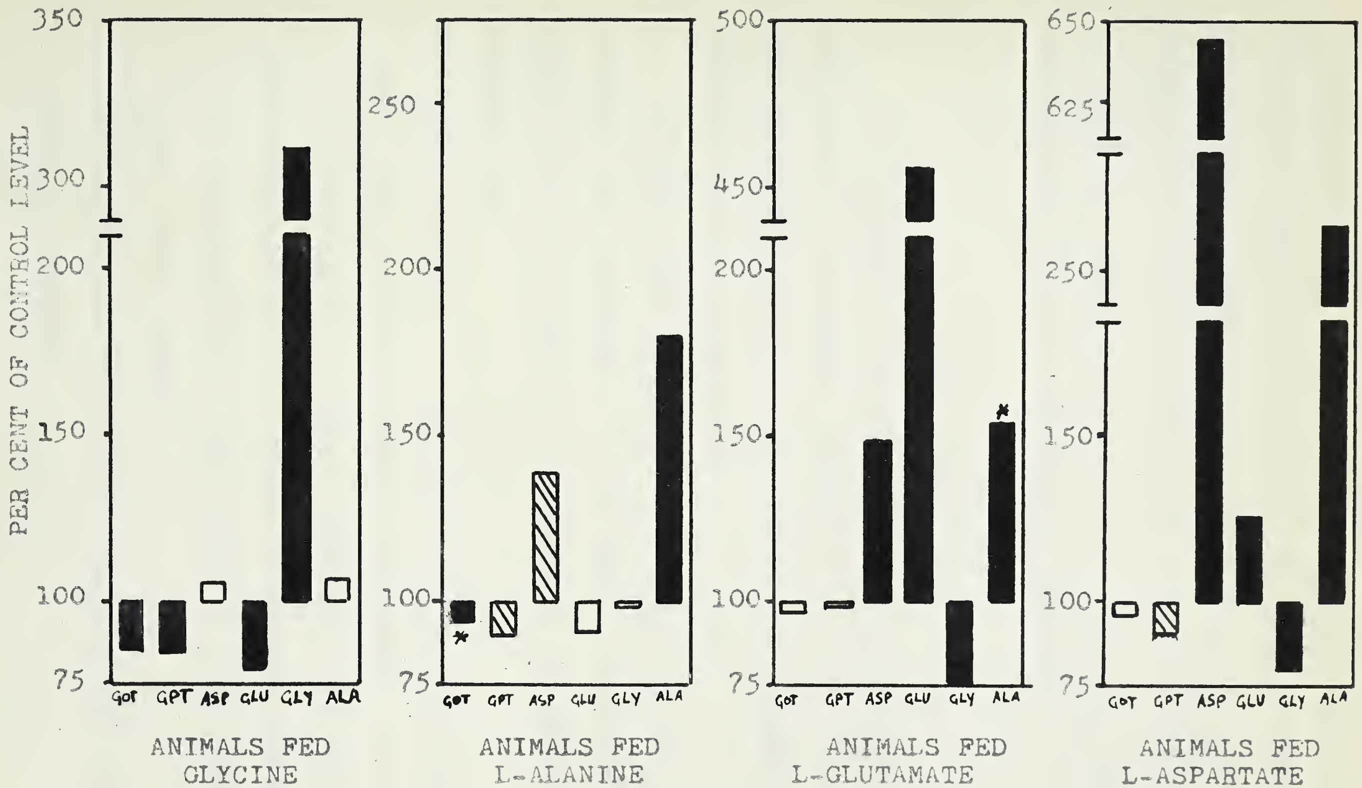


Fig. 45. The effect of ingestion of various amino acid solutions on the activity of intestinal GOT and GPT and on levels of free amino acids.

Black bars = statistically highly significant change. $p < 0.01$
Hatched bars = statistically significant change. $p < 0.05$
Empty bars = statistically insignificant change. $p > 0.05$
* $p < 0.02$

Force-feeding of 0.75 M L-aspartate: Similar results to those obtained when L-glutamate was fed, were obtained by feeding 0.75 M L-aspartate. No loss of GOT activity was found and the difference in the activity of GPT was significant only within the 5% level. The concentrations of L-aspartate, L-glutamate, and L-alanine all were increased and the concentration of glycine again was markedly depressed.

D. Discussion

Since little evidence has been reported concerning the metabolic mechanisms of intestinal cells, the above findings must be interpreted in terms of general mechanisms which involve several assumptions.

Several such "general mechanisms" can be employed to explain why intestinal transaminase activity should decrease when glycine, alanine, and aspartate are fed to two-day fasted rats.

1. Unphysiological conditions (produced by such situations as anoxia, the presence of abnormally high concentrations of a foreign or a toxic substance) can cause the cells to disintegrate or their walls to become permeable to protein molecules. The enzyme is then "washed away" by the blood stream.
2. The enzyme activity can be inhibited by the newly-introduced substance.

3. The compound which enters the cell can react with one or more of the substances already present in the cell to form a new compound which inhibits the enzyme activity.
4. When a tissue is in a state of starvation enzyme protein may break down and its hydrolysis products may react with the newly-introduced substance to form compounds which are required for the nutrition of the cell.

The data which were obtained as a result of feeding glycine can be explained most easily by the third mechanism. The data show that glycine ingestion is accompanied by decreased levels of intestinal glutamic acid. Hence, one might postulate that the glycine reacts with glutamic acid to form a compound which exerts an inhibitory effect on transaminase activity. As glutathione is known to be widely distributed in animal tissues it is probably safe to assume that an enzyme system which catalyzes glutathione synthesis exists in intestinal cells. This enzyme system may be the same as the one described by Bloch and his associates (86, 137) who showed that enzymic synthesis of glutathione by preparations from pigeon liver and from yeast involves two successive reactions:

glutamic acid + cysteine + ATP $\longrightarrow$ γ-glutamylcysteine + ADP + Pi

γ-glutamylcysteine + glycine + ATP $\longrightarrow$ glutathione + ADP + Pi

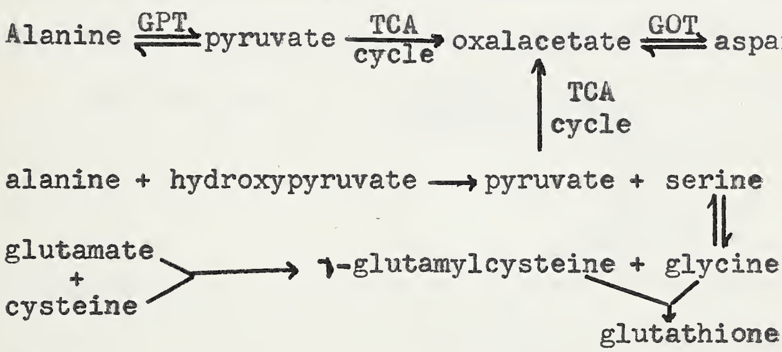
Waelsch and Rittenberg (147) have shown that the metabolic

turnover of glutathione in animal tissues and in yeast is extremely rapid, and it has been suggested that this widely distributed tripeptide may play a role in the biosynthesis of proteins. The intestinal mucosa, which exhibits the highest turnover rate of animal body proteins (49) as judged by isotope experiments, undergoes rapid cellular disintegration and replacement. Thus, it might be supposed that glutathione plays an extremely important role in the metabolism of mucosal cells. Finally, in an earlier chapter, we stated that an unknown substance always appeared on paper chromatograms when intestinal extracts were chromatographed. Although the spot has not been positively identified, Tuba and Neufeld (143) have provided evidence that the compound is glutathione. Judging by the color density of the spot, the concentration of the substance in intestine could be as high as 400 to 500 micrograms per gram of wet tissue. Hence, by making several assumptions, the results obtained by feeding glycine to fasted rats may be interpreted in the following way.

The requirement for glutathione in the intestine of a rat which has been starved for two days may be significant. When glycine is introduced into the cells, increased synthesis of glutathione may result. Glutamic acid and cysteine react to for γ-glutamylcysteine and this in turn combines with glycine to form glutathione. The glutamic

acid portion of γ-glutamylcysteine may compete with glutamic acid for the active sites on intestinal GOT and GPT and competitive inhibition could result.

When L-alanine was fed to fasted rats, inhibition of GOT and GPT again occurred. Along with the expected increase of the concentration of alanine, the concentration of L-aspartate was also found to be elevated. Alanine readily enters into a number of metabolic pathways, and the following schematic diagram demonstrates some of these.



Thus the increased concentration of aspartate which was found could be explained by transamination and involvement of the tricarboxylic acid cycle. The depression of transaminase activity, however, is not as simply interpreted. The schematic diagram shows a possible pathway for the formation of serine from alanine and hydroxypyruvic acid. This transamination reaction was reported by Sallach (1956) who postulated that hydroxypyruvic acid is an immediate precursor of serine and is formed from glucose via 3-phosphoglyceric acid. As the above diagram shows, inhibition of GOT and GPT again could be caused by γ-glutamylcysteine.

The results which were obtained when L-glutamate was fed to fasted rats can be explained on the basis of transamination. Since the levels of both aspartate and alanine were found to be elevated, it is obvious that this must be a result of GOT and GPT activity _in vivo_. In order for transamination to occur, however, pyruvate and oxalacetate are required. Glycine probably acts as the source of these α-keto acids, as a highly significant loss of this amino acid was found to occur. Pyruvate and oxalacetate probably were formed from glycine via the following mechanism:

$$\begin{tikzcd} \text{glycine} \arrow[r, shift left] & \text{serine} \arrow[l, shift left] \arrow[r, "\text{serine}", "\text{dehydrase}"'] & \text{pyruvate} \arrow[d, "\text{TCA cycle}"] + \text{formate} \\ & & \text{oxalacetate} \end{tikzcd}$$

The reversible conversion of glycine to serine has been reported by numerous investigators (41, 70, 125), while serine dehydrase has been found in mammalian liver by Chargaff and Sprinson (25) and Sayre and Greenberg (128).

Fig. 45 clearly illustrates that force-feeding of L-aspartic acid produces similar results to those obtained by feeding L-glutamic acid. The interpretation of the data, also, is similar and may be shown schematically as follows:

$$\begin{tikzcd} \text{aspartate} + \alpha\text{-ketoglutarate} \arrow[r, shift left, "\text{GOT}"] & \text{oxalacetate} + \text{glutamate} \arrow[l, shift left] \\ \alpha\text{-ketoglutarate} + \text{alanine} \arrow[u, dashed] \arrow[r, shift left, "\text{GPT}"] & + \ \text{pyruvate} \arrow[l, shift left] \\ \text{glycine} \arrow[r, shift left] & \text{serine} \arrow[l, shift left] \arrow[u] \end{tikzcd}$$

Thus, as long as sufficient glycine and aspartate are available, both transaminases continue catalyzing their respective reactions. Unlike glutamic acid, alanine is not removed from the reaction system. Hence, analysis of the intestine showed a greater per cent increase of alanine than of glutamic acid. The slight fall of GPT activity which was found may have resulted from competitive inhibition by the large concentration of aspartic acid.

E. Summary

1. Force-feeding of glycine to two-day fasted rats was shown to cause depression of intestinal GOT and GPT activity to 85% of control level. The concentration of intestinal glutamic acid was also found to be decreased. The relationship of glutathione synthesis to these findings was considered.
2. Depression of intestinal GOT and GPT activity also was observed as a result of force-feeding L-alanine. The concentrations of aspartate and alanine were found to be significantly elevated. Reference was made to two metabolic pathways for alanine which explain the increase in aspartic acid levels on the basis of transamination and involvement of the TCA cycle. Depression of GOT and GPT activity was attributed to competitive inhibition by γ-glutamylcysteine.
3. The results which were obtained by force-feeding L-glutamic acid confirmed earlier findings of Wiseman (150)

that glutamic acid undergoes transamination after being absorbed by rat intestine. There was no increase in the rate of synthesis of intestinal transaminases. A highly significant drop of glycine concentration was observed. This was explained on the basis of α-keto acid requirement as a result of increased transaminase activity. A possible pathway was shown whereby glycine is converted to pyruvate via serine.

4. When L-aspartate was fed to fasted rats, significant increases of both glutamate and alanine were found. In addition, the concentration of glycine was found to decrease to 80% of the control level. A cyclic transaminase system involving both GOT and GPT was suggested to explain the greater increase of alanine than of glutamic acid when aspartate is absorbed by rat intestine. The decrease of glycine concentration was explained as due to pyruvate formation via serine in order to provide substrate for the GPT reaction. A slight decrease of GPT activity was interpreted on the basis of competitive inhibition by aspartic acid.

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